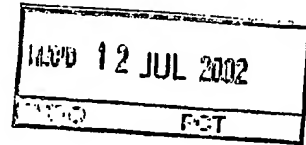




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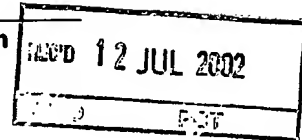
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#### FIELD OF THE INVENTION

(41)

The invention relates to the field of microbiology. In particular the invention relates to the field of identification of antiviral compounds.

5

#### BACKGROUND OF THE INVENTION

Several procedures are known in the art for the treatment of virus-related diseases and for the prevention of disorders that arise as a consequence of viral infections. Prophylactic vaccination is probably one of the most effective measures against potential fatal infectious diseases, since an individual can become fully or partly protected against new infections. The immune system makes sure that the virus that has entered is prevented from replicating in the individual and in most cases the virus disappears completely from the system. Therapeutic vaccination refers to the treatment of infected individuals intentionally to prevent the virus from replicating further and consequently to halt disease progression or to cure the disease by eliminating the virus from the body.

Another way of dealing with viral infections is through the use of antiviral agents that either attack the viral particle directly or that prevent the infection, propagation, replication, packaging and/or growth of the virus in the individual. These treatments are applied either when the individual has already been infected and so a prophylactic vaccination is obsolete, or when an individual is at immediate risk to encounter an infection. Antiviral molecules inhibit certain processes and phases in the viral life cycle, thereby inhibiting the virus from spreading.

Several *in vitro* and *in vivo* methods for the identification of such antiviral compounds are known in the art. Methods that make use of the specific antiviral activity of certain compounds include the plaque reduction assay, the yield reduction assay, the virus antigen determination assay, the dye-uptake assay, the cytopathic effect (CPE) determination assay and several *in vivo* assays for virus replication. Many of the *in vitro* methods, especially the plaque reduction assay, have the major disadvantage that they cannot be applied in (very) high-throughput screens. Although the plaque reduction assay can be applied for most viruses that are known to date, it is necessary to inoculate large numbers of susceptible cells in suitable conditions with ranges of virus titers as well as large ranges of antiviral compound titers to detect the correct concentration of the compound that significantly decreases the number of plaques. This makes the plaque reduction assay very suitable for measuring the right concentration of a specific compound that affects the growth of a particular virus but very unsuitable for the identification of such (new and unknown) compounds in a library. Since many of the molecule libraries consist of a very large collection of separate compounds ( $>10^{14}$  individual agents), it is required to have settings in which all separate compounds can be screened in a rapid and efficient manner with low costs. The other *in vitro* methods such as the yield reduction assay, the virus antigen determination assay, the dye-uptake assay and the cytopathic effect (CPE) determination assay are to a certain extent more suitable for high-throughput screening, but they clearly depend on the cell line that is used and whether such a cell line is able to grow in multi-well settings and for prolonged periods of time. Clearly, many of the primary cells that are used to determine the effect of an antiviral compound in plaque reduction assays cannot be cultured in high-

throughput settings, since these cells do not grow indefinitely. Evidently, the *in vivo* antiviral methods, such as for example the ferret-, the mouse- and chicken models for influenza infection (reviewed by Sidwell et al. 2000) are useless for the identification of novel compounds that prevent virus-cell recognition and virus infection, replication, propagation and growth, especially when high-throughput settings are preferred.

Many susceptible non-continuous cells have been identified in which most viruses propagate. As mentioned, these cells can be used in assays such as the plaque reduction assay but cannot be applied for screening of antiviral compounds, since they do either not grow in multi-well formats or they do not grow indefinitely. Only a limited number of continuous cell lines have been identified that support the growth of certain viruses. These cell lines include the green monkey VERO cells, the Madin-Darby Canine Kidney MDCK cells, the human lung embryo MRC-5 cells and the human A549 cells. However, a major drawback of these cells is that they only support the growth of a limited number of viruses, while not all of these cell lines are capable of continuous growth in multi-well formats. Nevertheless, a number of drugs displayed antiviral activities against viruses such as CMV, Influenza and HSV in the context of using the cells mentioned above. For example, Acyclovir, an approved purine nucleoside analogue, inhibited HSV replication in A549 cells (Li et al. 1988). Despite the few successful propagations of certain viruses on continuous cell lines and the prevention of propagation by a number of antiviral compounds, it was found that in many cases the cells did not support the complete life cycle of the mentioned viruses. This limits their use significantly in screening assays for antiviral compounds present in large libraries, because the life cycle of a virus is built up

from several phases in which a compound can have its point of impact.

Although many cell-based systems exist that can be applied for determining whether a certain compound is capable of preventing certain phases in the life cycle of a virus, no system is present in the art that combines the possibility of screening large numbers of (possible) antiviral compounds in a very high-throughput setting with the possibility of screening a large range of different viruses. No system is available in the art that combines these possibilities to determine the antiviral activity of a certain compound present in a compound library, in different phases of the life cycle of the particular virus that is attacked by this particular compound.

The present invention discloses novel uses of cell lines that fully support the complete life cycle of a very wide variety of pathogenic viruses and that provide methods for screening libraries of antiviral compounds for identification of molecules with antiviral activity that can interfere with the pivotal processes in any phase of the life cycle of a pathogenic virus. The fact that these cell lines support the infection, replication, propagation and growth of a large variety of viruses is disclosed in patent application PCT/NL00/00862, not yet published but incorporated herein by reference. PER.C6 cells are capable of growing in continuous cultures and have been cultured for over 200 passages. The fact that PER.C6 is suitable for high-throughput screenings is disclosed in WO 99/64582.

It is an object of the current invention to deal with a large number of the problems and drawbacks known from the art concerning the identification of antiviral compounds using the assays and cell lines described above.

## SUMMARY OF THE INVENTION

The current invention comprises methods that substantially lack above outlined drawbacks and that is characterized in methods for identifying a compound and/or determining whether said compound influences a phase in the life cycle of a virus comprising providing a cell with said compound and with at least a fragment of said virus sufficient for performing said phase and determining whether said phase is influenced in said cell, said cell comprising a nucleic acid encoding an adenovirus early protein or a functional part, derivative and/or analogue of said adenovirus early protein. Preferably, the methods provided by the invention comprise a virus selected from the group of adenoviruses, enteroviruses, herpes viruses, orthomyxoviruses, paramyxoviruses, retroviruses, rotaviruses, coronaviruses, flaviviruses, togaviruses, hepatitis causing viruses, pestiviruses, rhabdoviruses or Bunyaviridae viruses. Even more preferred, said virus is an essentially intact virus, wherein essentially is defined as comprising an intact coat capable of recognizing and infecting an appropriate host cell, further comprising nucleic acid capable of supporting all required functions for normal replication, propagation, packaging and releasing newly formed virus particles.

In a preferred embodiment the invention makes use of a human cell, said human cell being preferably derived from a kidney, a retina or from amniotic fluid. In an even more preferred aspect of the invention said human cell is transformed by an adenovirus early region 1 or -2 encoding nucleic acid, wherein said nucleic acid is preferably integrated in the genome of said cell.

The invention is further characterized in methods for determining whether a compound influences a phase in the life cycle of a virus comprising providing a cell with said compound and with at least a fragment of said

virus sufficient for performing said phase and determining whether said phase is influenced in said cell, said cell comprising a nucleic acid encoding an adenovirus early protein or a functional part, derivative and/or analogue of said adenovirus early protein, wherein  
5 determining whether a compound influences a phase in the life cycle of a virus comprises examining the activity and/or amount of a cellular protein and/or examining the interaction of said virus with said cell and/or examining  
10 the activity and/or amount of said virus or a fragment thereof and/or examining the viability of said cell.

The invention is further characterized in uses of a cell, said cell comprising nucleic acid encoding an adenovirus early protein, such as early region 1 or -2,  
15 for screening a library of compounds for the presence of a compound capable of influencing a phase in the life cycle of a virus capable of entering said cell. In a preferred aspect of the invention, said uses of a cell comprise a virus selected from the group of adenoviruses,  
20 enteroviruses, herpes viruses, orthomyxoviruses, paramyxoviruses, retroviruses, rotaviruses, coronaviruses, flaviviruses, togaviruses, hepatitis causing viruses, pestiviruses, rhabdoviruses or  
Bunyaviridae viruses.

25 In a preferred embodiment of the present invention said compound is present as part of a compound library. In a more preferred embodiment said compound library is used in high-throughput settings. In an even more preferred embodiment of the present invention said method  
30 comprises isolating said compound.

In another aspect, the invention provides methods for identifying a compound with antiviral activity comprising providing a cell from a first collection of cell cultures with at least a fragment of a first virus,  
35 said fragment capable of performing a phase in the life cycle of said first virus, providing said cell from a



first collection of cell cultures with a compound and determining whether said compound is capable of influencing said phase in the life cycle of said first virus, said method further comprising providing a cell  
5 from a second collection of cell cultures with at least a fragment of a second virus, said fragment capable of performing a phase in the life cycle of said second virus, providing said cell from a second collection of cell cultures with a second compound and determining  
10 whether said second compound is capable of inhibiting said phase in the life cycle of said second virus, wherein said cells from said first and said second collection comprise a nucleic acid encoding an adenovirus early protein and wherein said first and said second  
15 library of compounds may be the same or different.

In another aspect, the invention provides methods for determining the effect of a compound on a phase in the life cycle of a virus comprising the steps of culturing a cell, said cell otherwise capable of  
20 supporting said phase in the life cycle of a virus, in the presence of said virus under conditions otherwise conducive to said phase in the life cycle of said virus and in the absence of said compound, examining the effect of the absence of said compound on said phase in the life  
25 cycle of said virus. Preferably, said compound is a natural constituent of said cell. More preferably, said natural constituent is a receptor protein, or a fragment thereof, for said virus. Even more preferably, said cell is comprised in a set of clones of cells or a library of  
30 cells, said cells comprising a gene being effectively blocked from being expressed, wherein effectively blocked is defined as significantly reduced. In an even more preferred aspect of the invention, said cell comprises a nucleic acid encoding an adenovirus early protein or a  
35 functional part, derivative and/or analogue of said adenovirus early protein.

## SHORT DESCRIPTION OF THE FIGURES

Fig.1. Percentage of infected cells scored microscopically after immunofluorescence assay. Samples  
5 derived from infection at multiplicity of infection (moi) 10 and 1, at 48 h post infection are not shown, because of full CPE.

Fig.2. Percentage of infected cells (positive cells)  
10 scored microscopically after immunofluorescence assay versus percentage of dead cells measured via FACS after propidium iodide staining, at moi's of  $10^{-3}$  (A) and  $10^{-4}$  (B). Poor viability of the cells from samples derived from infection at moi  $10^{-3}$  did not give rise to reliable  
15 data.

Fig.3. Kinetics of virus propagation measured in hemagglutinating units (HAU) from day 1 to day 6 after infection.  
20

Fig.4. Percentage of infected cells (positive cells) viewed microscopically after immunofluorescence assay. (A) A/Beijing/262/95 and (B) X-127.

25 Fig.5. Kinetics of virus propagation measured in HAU from day 1 to 6 after infection. (A) A/Beijing/262/95 and (B) X-127.

Fig.6. Infection with A/Sydney/5/97 on PER.C6. (A) Effect  
30 of trypsin-EDTA on HAU titers. (B) HA concentration in  $\mu\text{g/ml}$  and (C) virus infectivity titers in plaque forming units per ml (pfu's/ml) as measured in crude viral supernatants, 96 hours post infection.

35 Fig.7. Infection with B/Harbin/7/94 on PER.C6. (A) Effect of different concentrations of trypsin-EDTA present

during and after virus infection on growth kinetics. (B) HAU titers per 50  $\mu$ l and (C) virus infectivity titers in pfu/ml.

- 5 Fig.8. Infection with X-127 using an moi of  $10^{-3}$  on PER.C6. (A) Effect of trypsin-EDTA on HAU given in HAU/50  $\mu$ l and (B) virus infectivity titers in pfu/ml during 5 days after infection.
- 10 Fig.9. Infection with X-127 using an moi of  $10^{-4}$  on PER.C6. (A) Effect of trypsin-EDTA on HAU given in HAU/50  $\mu$ l and (B) virus infectivity titers in pfu/ml during 5 days after infection.
- 15 Fig.10. Electron micrographs of Influenza A/Sydney/5/97. (A) PER.C6 cells 72 hrs post infection. (B and C) Negative staining on virus derived from infected PER.C6. (D and E) Negative staining of sucrose purified material.
- 20 Fig.11. (A) Different Influenza A and B strains tested on PER.C6 cells. (B) Infectivity titers of three depicted A- and B-type Influenza viruses derived from infected PER.C6 cells.
- 25 Fig.12. Immunofluorescence of PER.C6 and Vero cells infected with viruses other than Influenza. (A) Positively staining cells upon infection with Measles virus. (B) Positively staining cells upon infection of Vero cells with HSV-1 virus. (C) Positively staining  
30 cells upon infection of Vero cells with HSV-2 virus. (D) Positively staining cells upon infection of PER.C6 cells with HSV-1 virus. (E) Positively staining cells upon infection of PER.C6 cells with HSV-2 virus.

Fig.13. Infectivity titers determined after propagation of Measles virus (A), HSV-1 (B) and HSV-2 (C) virus on PER.C6 cells.

- 5 Fig.14. Replication of Rotavirus after infection of PER.C6 (A) and Vero (B) cells with different moi's as measured by ELISA in crude supernatants.

- 10 Fig.15. Toxicity of acyclovir on PER.C6 as measured by MTS assay after 6 days of incubation of the cells in the presence of different dilution of the compound as indicated.

- 15 Fig.16. Virustatic activity of acyclovir for Herpes Simplex Virus type 1 and type 2 (HSV-1 and HSV-2) using PER.C6 cells as a substrate for virus replication and TCID<sub>50</sub> calculations based on CPE score on day 6 post infection.

- 20 Fig.17. Virustatic effect of acyclovir against (A) HSV-1 and (B) HSV-2 using infection of PER.C6 with different 10 fold serial dilutions of virus and viable cell scoring by MTS assay on day 6 post infection.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel methods for determining whether a compound influences a phase in the life cycle of a virus comprising providing a cell with said compound and with at least a fragment of said virus sufficient for performing said phase and determining whether said phase is influenced in said cell, said cell comprising a nucleic acid encoding an adenovirus early protein or a functional part, derivative and/or analogue of said adenovirus early protein. The phase in the life cycle of a virus is defined as a phase that can comprise a step or a period during which a virus recognizes its host cell or during which the virus interacts with its host cell through a protein, preferably a protein expressed by the host cell. Said phase in the life cycle of a virus is further defined as a phase that can comprise a step or a period during which a virus infects (enters) a cell, replicates its nucleic acid with or without using cellular factors, and produces the required proteins in said cell for propagation and/or packaging of the newly made viral particle(s) in said cell. A life cycle of a virus can be generally defined as

- (a) recognition and/or interaction of the virus with a host cell,
- (b) entering (infecting) said host cell,
- (c) changing the host cell environment to enable generation of new virus particles, for instance by expression of viral proteins from the infected nucleic acid,
- (d) replication of the nucleic acid,
- (e) expression of viral proteins required for packaging new viral particles,
- (f) packaging of new viral particles and
- (g) release of the newly formed particles from the infected cell,

wherein these phases can occur sequentially or simultaneously and wherein several sub-phases might be distinguished.

To determine to what extent a certain compound  
5 influences a specific phase in the life cycle of a virus,  
several methodologies can be applied. Examples of such  
methodologies are (1) measuring the levels and/or  
activities of viral and/or cellular proteins that are  
present upon entry of the virus into the host cell, (2)  
10 examining the recognition of the virus with the host cell  
or the interaction of the virus with the host cell and/or  
host cell proteins, (3) examining the activity and/or  
amount of the virus or a fragment thereof and (4)  
examining the viability of the infected host cell.

15 In another aspect the invention provides the use of  
a cell, said cell comprising nucleic acid encoding an  
adenovirus early protein, for screening a library of  
compounds for the presence of a compound capable of  
influencing a phase in the life cycle of a virus capable  
20 of entering said cell.

In another aspect the invention provides novel  
methods for identifying a compound with antiviral  
activity comprising providing a cell with at least a  
fragment of a virus, said fragment capable of performing  
25 a step in the life cycle of said virus, providing said  
cell with a compound and determining whether said  
compound is capable of influencing said step in the life  
cycle of said virus, wherein said cell comprises a  
nucleic acid encoding an adenovirus early protein or a  
30 functional part, derivative and/or analogue of said  
adenovirus early protein.

The viruses used by the present invention to screen  
for antiviral compounds comprise an adenovirus, an  
enterovirus (such as rhinovirus, aphtovirus, or  
35 poliomyelitisvirus), a herpesvirus (such as herpes  
simplex virus, pseudorabies virus or bovine herpes

virus), an orthomyxovirus (such as Influenza virus), a paramyxovirus (such as newcastle disease virus, respiratory syncytio virus, mumps virus or measles virus), a retrovirus (such as human immunodeficiency virus), a parvovirus, a papovavirus, a rotavirus, a coronavirus (such as transmissible gastroenteritis virus), a Flavivirus (such as tick-borne encephalitis virus or yellow fever virus), a togavirus (such as rubella virus or eastern-, western-, or venezuelean equine encephalomyelitis virus), a hepatitis causing virus (such as hepatitis A, -B or -C virus), a pestivirus (such as hog cholera virus), a rhabdovirus (such as rabies virus) or a Bunyaviridae virus (such as Hantavirus).

15       The invention relates to the identification of antiviral compounds directed against viruses or viral pathogens that previously could not or poorly be grown on human cells *in vitro*. The invention is particularly useful for the production of therapeutics to aid in protection against viral pathogens for vertebrates, in particular mammals and especially humans.

20       The invention discloses methods and means for the identification and/or validation of compounds harbouring antiviral activities and the like that interfere with the life cycle of viral pathogens. The identification and/or validation can either be performed in low-throughput, medium-throughput, high-throughput or ultra high throughput settings, ranging from a few compounds to  $>10^{14}$  compounds present in a library of compounds. The invention relates to the screening of compound libraries comprising non-proteinaceous substances like small or large (synthetic) compounds such as nucleoside analogues or nucleic acid such as DNA molecules. The invention also relates to the screening of compound libraries comprising molecules like proteinaceous substances such as peptides, antibodies, hormones, receptors or other protein binding

entities or fragments thereof. The non-proteinaceous substances, proteinaceous substances in such collections can be expressed on phage particles or can be present as separate molecules not expressed on phage particle. The invention moreover relates to the screening of compound libraries comprising large particles like viruses, virus particles, phage and fragments thereof. The compounds can have already known- or yet undiscovered functions for antiviral activity.

The invention is particularly useful for the identification of molecules that interfere with infection of cells by viral pathogens, binding and/or entry of viral pathogens into cells, replication of said viral pathogens in cells, packaging of viral pathogens into viral particles and/or release of said viral pathogens from cells and/or re-infection of cells by such viral pathogens, to aid in protection against viral pathogens for vertebrates, in particular mammals and especially humans.

The invention relates to the use of mammalian cells, preferably a non-tumour-derived transformed human cell for the screening of such libraries of molecules. More preferably the invention makes use of a human non-tumour derived cell that is transformed with early proteins of Adenovirus. Even more preferred is the use of human cells that are not derived from tumours and that are transformed with early region E1 from adenovirus, such as the human embryonic retina derived PER.C6 cells (ECACC number 96022940). The present invention discloses human immortalized cell lines (PER.C6 and derivatives thereof) that are generally used for the production of recombinant adenoviral vectors and for the purpose of propagating, production and harvesting viruses other than adenovirus to generate vaccines (disclosed in patent application PCT/NL00/00862, not yet published). The PER.C6 cells were generated by transfection of primary human embryonic



retina cells, using a plasmid that contained the Ad serotype 5 (Ad5) E1A- and E1B-coding sequences (Ad5 nucleotides 459-3510) under the control of the human phosphoglycerate kinase (PGK) promoter.

5       The following features make PER.C6 and derivatives thereof particularly useful as a host for virus production: it is a fully characterized human cell line, it was developed in compliance with GLP, it can be grown as suspension cultures in defined serum-free medium,  
10 devoid of any human or animal serum proteins; its growth is compatible with roller bottles, shaker flasks, spinner flasks and bioreactors, with doubling times of about 35 h. Moreover, it was found that PER.C6 were very suitable for growth in multi-well settings and for the propagation  
15 of adenoviruses in high-throughput methodologies (WO 99/64582).

      The invention provides the use of such cell lines for the identification of novel antiviral compounds that act towards viruses for which no known antiviral  
20 compounds were thus far identified. Moreover, the invention enables the identification of known antiviral compounds, which also act upon viruses that could thus far not be propagated on cell lines used in the art. Such known and unidentified antiviral compounds (often small  
25 molecules) can either be rationally designed or identified in screenings assays. The antiviral strategies that these compounds employ are sometimes known (e.g. neuraminidase inhibitors for treatment of influenza infection and DNA synthesis inhibitors for the treatment  
30 of HSV infection). On the other hand, many drugs have been developed that exert an antiviral effect in patients through mechanisms that are not completely understood. Many of such drugs were identified in recent years.

      For example the protein Interferon alpha is used for  
35 the treatment of HCV infected patients. It is applied alone or in combination with Ribavirin (Keating 1999),

although problems have occurred for HCV treatment when used alone (Polyak et al. 2000). Famciclovir and valaciclovir are two approved compounds and that are used for Herpes Simplex Virus (HSV) and for varicella-zoster infections (Keating 1999). Ganciclovir (GCV), a purine nucleoside analogue was approved to treat CMV retinitis (Keating 1999; Alrabiah et al. 1996), while another approved purine nucleoside analogue, Acyclovir (ACV) is being applied for genital herpes, HSV encephalitis, mucocutaneous herpetic infections, varicella-zoster and for herpes-zoster infections (Keating 1999; Dwyer et al. 1997). Two nucleoside phosphonate analogues, Adefovir and Tenofovir, are being used to treat HBV infections (Ying et al. 2000). The compound Dibenzofuran was found to block rhinovirus replication *in vitro* and to hinder the cytopathic effect in cells infected with HRV14 or HRV16 (Murray et al. 1999). In recent years also several protease inhibitory agents were identified that were active against the Human Immunodeficiency Virus (HIV): Saquinavir (Figgitt et al. 2000) and Nelfinavir (Bardsley-Elliott et al. 2000a). Nevirapine is an approved non-nucleoside Reverse Transcriptase inhibitor (NNRTI) used against HIV infections (Bardsley-Elliott et al. 2000b), while a non-nucleoside RT inhibitor (NNRTI), Delavirdine (a bisheteroarylpiperazine derivative) was also found to be active against HIV (Scott et al. 2000; Joly et al. 2000). Amantidine and Rimantadine are two drugs that are used for the treatment of Influenza A infections (Keating 1999). Nucleoside analogues are reviewed by Pastor-Anglada et al. (1998) and the idea to use protease inhibition to attack virus infections is discussed by Todd et al. (2000). This list of antiviral agents is by far not limiting and many proteins, small molecules and other types of compounds are still in several different phases of development.

Although the PER.C6 cell line was developed for the production of recombinant adenoviral vectors (mainly derived from Adenovirus serotype 5, or Ad5) through the complementation of E1 deleted vectors by the E1 functions provided by the integrated E1-expressing plasmid, it was also found that PER.C6 cells were able to support the production of exogenous proteins such as recombinant human Erythropoietin (EPO) or human monoclonal antibodies encoded by expression plasmids that were transfected and integrated stably or that were present in a transient system (WO 00/63403). Besides Ad5, PER.C6 is also able to support the growth of every other Adenoviral serotype found to date (a total of 51 Adenovirus serotypes, disclosed in European patent application publication number EP 0978566).

Interestingly, PER.C6 also supports the growth of an entire different set of viruses besides Adenoviruses. Examples of these viruses are, but are not limited to Human- and Duck Influenza virus, Human- and Rhesus Rotavirus, Measles Virus, Respiratory Syncytium virus A and B, Parainfluenza types -1, -2 and -3, Poliovirus types -1, -2 and -3, Coxsackie virus B2, B4, A9, Echovirus types -4, -7 and -11, Japanese encephalitis virus, Hantavirus and Herpes Simplex Virus types -1 and -2. Examples of such viruses that can grow on PER.C6 are disclosed herein and in PCT/NL00/00862 (incorporated herein by reference).

For example, rotaviruses can grow on PER.C6 cells. Rotaviruses, members of the family of Reoviridae, are double strand RNA viruses consisting of 11 RNA segments, each coding for a structural or non-structural viral protein (VP). Given the worldwide prevalence of rotavirus associated infant morbidity and mortality, large scale vaccination against Rotavirus is thus far considered the most effective way to combat this virus. The goal of vaccination would not be to prevent the disease but to

reduce its severity and complication, especially during the first few years of life. The only vaccine at present is a live attenuated orally delivered composition that is associated with intussusception, a bowel obstruction disease. For that reason this vaccine is no longer in use. It is evident that there is a need for anti-rotavirus drugs that can be used to treat Rotavirus infected patients. The identification of such drugs was thus far hampered by the difficulty of growing the virus in suitable systems. The present invention provides a human cell line that is highly capable of supporting infection, replication, propagation and growth of rotavirus, thereby providing a system for the identification of compounds that prevent rotavirus generation in human cells using a multi-well set up.

Adenoviruses are another set of viruses for which the present invention provides the possibility to identify antiviral compounds against. Although Adenoviruses are known to cause minor disorders such as common colds, it is also known that in immuno-suppressed patients adenoviruses can cause severities that can even lead to death of such patients. Of all bone-marrow transplant patients that die during or after treatment, approximately 20% dies of an adenovirus infection. Clearly, there is a great need for anti-adenoviral compounds that can prevent the occurrence of malignancies due to adenoviral infections in these patients that have a suppressed immune response.

## EXPERIMENTAL PROCEDURES

### PER.C6 and MDCK Cell culture

Madin-Darby Canine Kidney (MDCK) cells and PER.C6  
5 cells (deposited under No. 96022940 at the ECACC,  
described in WO 97/00326 and US patent 6,033,908) were  
cultured in Dulbecco's modified Eagle's medium (DMEM,  
Life Technologies) containing 10% heat inactivated fetal  
bovine serum and 1x L-Glutamin (Gibco), at 37°C and 10%  
10 CO<sub>2</sub>. Suspension cultures of PER.C6 were cultured in ExCell  
525 (JRH Biosciences) supplemented with 1x L-Glutamin, at  
37°C and 10% CO<sub>2</sub>, in stationary cultures in 6 well dishes  
(Greiner) or in 490 cm<sup>2</sup> tissue culture roller bottles  
(Corning Costar Corporation) during continuous rotation  
15 at 1 rpm.

### Immunofluorescence test

Direct immunofluorescence assays for the detection  
of Influenza virus infection were carried out using the  
20 IMAGEN<sup>TM</sup> Influenza Virus A and B kit (Dako) according to  
the standard protocol of the supplier. Samples were  
viewed microscopically using epifluorescence  
illumination. Infected cells were characterised by a  
bright apple-green fluorescence.

25

### Propidium Iodide staining

Cell pellets were resuspended in 300 µl of cold  
PBS/0.5% BSA + 5 µl of propidium iodide (concentration 50  
µg/ml) in PBS/FCS/azide solution known to persons skilled  
30 in the art. Viable and dead cells were then detected via  
flow cytofluorometric analysis.

### Haemagglutination assay

In general, haemagglutination assays for Influenza  
35 virus titers were performed according to methods known to  
persons skilled in the art. Here, 50 µl of a two-fold

diluted virus solution in PBS was added to 25  $\mu$ l PBS and 25  $\mu$ l of a 1% suspension of turkey erythrocytes (Biotrading Benelux B.V.) in PBS and incubated in 96 well microtiter plates at 4°C for 1 h. The haemagglutination pattern was examined and scored, and expressed as hemagglutinating units (HAU's). The number of HAU's corresponded to the reciprocal value of the highest virus dilution that showed complete haemagglutination.

#### 10 Western blot analysis of the Influenza HA protein.

In general, obtained Influenza viruses were disrupted in a Laemmli buffer according to methods known to persons skilled in the art and different volumes of obtained protein mixtures were separated using 10% SDS/PAGE gels. In brief, blots were blocked for 30 min at room temperature with block solution (5% non fat dry milk powder (Biorad) in TBST supplemented with 1% rabbit serum (Rockland)), followed by 3 washes with TBST. Then, the blots were incubated with the anti A/Sydney/5/97 HA antiserum (98/768 NIBSC) diluted 1/500 in 1%BSA/TBST with 5% rabbit serum (Rockland) O/N at room temperature. Again the blots were washed 8 times with TBST. Finally the blots were incubated with the rabbit anti sheep antiserum (HRP labelled, Rockland) 1/6000 diluted in block solution for 1 h at room temperature. After 8 washes with TBST the protein-conjugate complex was visualised with ECL (Amersham Pharmacia Biotech), and films (Hyperfilm, Amersham Life Science) were exposed. The antisera were obtained from the NIBSC (UK) and applied in dilutions recommended by the NIBSC.

#### Single Radial Immunodiffusion (SRID) assay

The concentration of haemagglutinin in supernatants, derived from Influenza virus infected-PER.C6 cells, was determined by the single radial immunodiffusion (SRID) test as previously described (Wood et al 1977). The assay

was performed using standard NIBSC (UK) antigens and antisera reagents.

#### Plaque assay

5        A total of 1 ml of 10-fold serially diluted viral supernatants were inoculated on MDCK cells which were grown until 95% confluence in 6-well plates. After 1 h at 35°C the cells were washed twice with PBS and overloaded with 3 ml of agarose mix (1.2 ml 2.5% agarose, 1.5 ml 2x  
10 MEM, 30 µl 200 mM L-Glutamine, 24 µl trypsin-EDTA, 250 µl PBS). The cells were then incubated in a humid, 10% CO<sub>2</sub> atmosphere at 35°C for approximately 3 days and viral plaques were visually scored.

#### 15 Virus infectivity assay (TCID<sub>50</sub>)

Titration of infectious virus was performed on MDCK cells. In brief, cells were seeded in 96 well plates at a density of  $4 \times 10^4$  cells/well in DMEM supplemented with 2 mM L-Glutamin. Twenty-four hours later cells were infected  
20 with 100 µl of ten fold serially diluted culture supernatants, in quadruplicate, in medium containing Trypsin-EDTA at the concentration of 4 µg/ml. Two hours after infection cell monolayers were washed two times in PBS and incubated in medium containing trypsin for 7  
25 days, at 35°C. Supernatants from these cultures were then tested in an HA assay. TCID<sub>50</sub> titers were calculated according to the method of Karber (1931), which is well known to persons skilled in the art.

## EXAMPLES

To illustrate the invention, the following examples are provided, not intended to limit the scope of the invention.

### Example 1

#### **PER.C6 cells as permissive cell line for Influenza A virus**

It was not known prior to the invention described in PCT/NL00/00862 (not published), that PER.C6 as a human cell could sustain Influenza virus infection and replication. It was verified whether PER.C6 cells are permissive for Influenza virus infection in comparison with the dog cell line Madin-Darby Canine Kidney (MDCK), which served as a positive control.

On the day before infection,  $2 \times 10^5$  MDCK cells per well were seeded in 6-well plates. Twenty four hours later,  $4 \times 10^5$  seeded PER.C6 and the MDCK cells per well were infected with the H1N1 strain A/Puerto Rico/8/34 (titer  $3.6 \times 10^7$  pfu/ml), (obtained from Dr. E. Claas, Leiden University Medical Centre, The Netherlands). Infection was performed at various multiplicities of infection (moi's) ranging from 0.1 to 10 pfu/cell. After about 2 h of incubation at 37°C, the inoculum was removed and replaced by fresh culture medium. A direct immunofluorescence assay for the detection of Influenza virus infection was performed 24 h and 48 h post infection. The experiment showed permissiveness of PER.C6 for Influenza infection, with percentages of positive cells moi-dependent and comparable with MDCK (Fig.1).

### Example 2

#### **PER.C6 used for Influenza A virus propagation.**

It was verified whether not only permissiveness but also replication and propagation of Influenza virus could



be supported by PER.C6. On the day of infection, PER.C6 cells were seeded in 490 cm<sup>2</sup> tissue culture roller bottles, with the density of  $2 \times 10^5$  cells/ml in a final volume of 40 ml, in the presence of 5 µg/ml of trypsin-  
 5 EDTA (Gibco-BRL). Cells were either mock inoculated or infected with the H3N2 strain A/Shenzhen/227/95 (titer  $1.5 \times 10^6$  pfu/ml) (obtained from Dr. E. Claas, Leiden University Medical Centre, The Netherlands). Infections were performed at moi  $10^{-4}$  and  $10^{-3}$  pfu/cell. After 1 h of  
 10 incubation at 37°C, the inoculum was removed by spinning down the cells at 1500 rpm and resuspending the cells in fresh culture medium + 5 µg/ml of trypsin-EDTA. Harvest of 1.3 ml of cell suspension was carried out each day, from day 1 to day 6 post-infection. Supernatants were  
 15 stored at -80°C and used for haemagglutination assays. Cell pellets were used for direct immunofluorescence tests and for propidium iodide staining. Results of these experiments are shown in Fig.2 and Fig.3 respectively.

### 20 **Example 3**

#### **Permissiveness of PER.C6 for different Influenza strains**

To further investigate the permissiveness of PER.C6 for propagation of various Influenza strains, an infection by using the H1N1 vaccine strains  
 25 A/Beijing/262/95 and its reassortant X-127, obtained from the National Institute for Biological Standards and Control (NIBSC, UK) was performed. On the day of infection, PER.C6 cells were seeded in 490 cm<sup>2</sup> tissue culture roller bottles, with the density of approximately  
 30  $1 \times 10^6$  cells/ml in a final volume of 50ml. Cells were inoculated with 5 µl ( $10^{-4}$  dilution) and 50 µl ( $10^{-3}$  dilution) of virus in the presence of 5 µg/ml trypsin-EDTA. In order to establish if trypsin was indeed required, one more infection was carried out by  
 35 inoculating 5 µl of the strain A/Beijing/262/95 in the absence of the protease. After approximately 1 h of

incubation at 37°C, the inoculum was removed by spinning down the cells at 1500 rpm and resuspending them in fresh culture medium  $\pm$  5  $\mu$ g/ml of trypsin-EDTA. At day 2 and day 4 post-infection more trypsin was added to the samples. Harvest of 1.3 ml of cell suspension was carried out from day 1 to day 6 post-infection. Supernatants were stored at -80°C and used for haemagglutination assays and further infections; cell pellets were used for direct immunofluorescence tests. Results obtained with the above mentioned immunofluorescence and haemagglutination assays are shown in Fig.4 and Fig.5, respectively, illustrating the efficient replication and release of the viruses.

#### Example 4

Effect of different concentrations of trypsin-EDTA on the viability of PER.C6 cells, on the Influenza virus production in PER.C6 cells and on the HA protein derived thereof.

Due to the absolute trypsin requirement for the propagation of Influenza viruses in cell cultures, the effects of different concentrations of trypsin-EDTA on PER.C6 cell viability and virus replication in PER.C6 cells, after infection with several Influenza strains, were investigated.

25

#### Infection with Influenza virus strain A/Sydney/5/97 in the presence of low concentrations of trypsin

On the day of infection, PER.C6 cells were seeded in 490 cm<sup>2</sup> tissue culture roller bottles, at a density of  $1 \times 10^6$  cells/ml, in the presence of trypsin-EDTA at final concentrations of 0.5, 1, 2, 3 and 5  $\mu$ g/ml.

These trypsin concentrations did not interfere with the growth characteristics of the cells and their viability (data not shown). Cells were either, mock infected or infected with PER.C6-grown Influenza virus A/Sydney/5/97

at an moi of  $10^{-4}$  pfu/cell. The viral production was monitored by direct immunofluorescence (data not shown), haemagglutination assays, single-radial-immunodiffusion (SRID) and plaque assays, all as described above. Results from this experiment are depicted in Fig.6 and show that the HA content as measured by SRID as well as the biological activity of the virus, expressed in HAU, were highest when a trypsin concentration of 1  $\mu$ g/ml was used. Fig.6 also shows that by using a plaque assay the highest number of plaque forming units (pfu) per ml was observed in the sample corresponding to cells grown in medium containing 2  $\mu$ g/ml of trypsin.

Infection with Influenza virus strain B/Harbin/7/94.

On the day of infection PER.C6 cells were seeded in 490 cm<sup>2</sup> tissue culture roller bottles at a density of  $1 \times 10^6$  cells/ml, in the presence of different concentrations of trypsin-EDTA, ranging from 1 to 5  $\mu$ g/ml. Cells were infected with PER.C6-grown virus B/Harbin/7/94 at an moi of  $10^{-3}$  pfu/cell. Production of the virus was monitored by direct immunofluorescence, haemagglutination and plaque assays as shown in Fig.7. The infectability of PER.C6 at day 2 increased with the concentration of trypsin. At day 3 however, no significant difference was observed in the percentage of infected cells when 1, 2.5 or 5  $\mu$ g/ml trypsin was present. In the absence of trypsin (0  $\mu$ g/ml) no Influenza virus infection was detected. At the day of the last harvest (day 4 post-infection), the biological activity of the virus, as measured by haemagglutination assay, did not differ significantly. Interestingly, the infectivity assay performed in samples that were taken at day 3 and 4 after infection, showed a difference in the production of the virus. The highest titers were obtained at day 3 and

day 4 when a trypsin concentration of 2.5 to 5 (day 3) and 1 µg/ml (day 4) were used.

Infection with Influenza virus reassortant X-127.

5 On the day of infection, PER.C6 cells were seeded in T25 tissue culture flasks, at a density of  $1 \times 10^6$  cells/ml, in the presence of different concentrations of trypsin-EDTA ranging from 0 to 7.5 µg/ml. Cells were infected with PER.C6-grown virus X-127 (egg-reassortant for the  
10 strain A/Beijing/262/95) at an moi of  $10^{-4}$  and  $10^{-3}$  pfu/cell. Viral growth was monitored by direct immunofluorescence, haemagglutination and plaque assays. As shown in Fig.8 and Fig.9, HAU titers were identical between samples, independent of the trypsin concentration  
15 and the initial moi that was used. Furthermore, no significant differences were observed in the infectivity titers, as measured by plaque assay.

**Example 5**

20 **Electron microscopy analysis of Influenza viruses on PER.C6 cells.**

Transmission electron microscopy studies were done on PER.C6 cells that were infected with the Influenza strain A/Sydney/5/97 as well as on viral containing  
25 supernatants and sucrose purified material to determine the phenotype of this Influenza virus produced on PER.C6. All methods that were used are well known to persons skilled in the art. Fig.10 shows that the last stages of the virus life cycle are represented by budding and  
30 release of enveloped virions from the cytoplasmic membrane. Spikes corresponding to the HA and NA viral proteins were detected, ornamenting the periphery of the virion particles. The figure also shows the characteristic pleiomorphism of Influenza viruses.

**Example 6****Infection of PER.C6 with a large variety of Influenza A and B virus strains**

Static suspension cultures of PER.C6 cells that were  
 5 grown in T25 flasks and/or in 6 well plates in ExCell 525  
 medium, were infected at a cell density of  $10^6$  cells/ml  
 with 16 different strains of Influenza viruses shown in  
**Fig.11A**. These strains comprised several H3N2, H1N1, B  
 type and Avian strains. Infections were performed in the  
 10 presence of 5  $\mu$ g/ml of trypsin. The viruses were obtained  
 from NIBSC (UK) as egg-passaged wild type or reassortant  
 strains and are noted in. Infection was performed with a  
 virus dilution recommended by the NIBSC in the product  
 sheets that were delivered with the different strains.  
 15 All viruses tested were capable of propagation on PER.C6  
 as visualized by immunofluorescence (data not shown) and  
 titration of supernatant fluids in pfu assay (**Fig.11B**).

These results show that even Influenza strains  
 (depicted by an asterisks), such as A/Johannesburg/33/94,  
 20 B/Beijing/184/93 and A/Duck/Singapore-Q/F119-3/97, can  
 replicate and be produced on the human PER.C6 cells.

**Example 7****Generation of Herpes Simplex type 1 (HSV-1) virus, Herpes  
 25 Simplex type 2 (HSV-2) virus and Measles virus on PER.C6.**

It was tested whether other viruses than Influenza  
 virus and Adenovirus, such as Herpes simplex virus type 1  
 and 2 and Measles virus could also replicate on PER.C6.  
 Vaccines that are derived from these PER.C6-grown viruses  
 30 and that induce neutralizing effects in humans for  
 protection against wild type infections are generated  
 from the PER.C6-grown virus batches. The strains that  
 were obtained from ATCC and used for infection of PER.C6  
 cells are depicted in **Table I**.

**Table I**

Herpes simplex virus and Measles strains that were obtained from the ATCC and that were used for infection of PER.C6 cells.

5

Virus	Strain	ATCC catnr.	Lotnr.	Passage history	Titer
Herpes Simplex Type 1 (HSV-1)	Macintyre	VR-539	1327850	y.s./12, PR RabK/5, Mb/1, PrRabK/5, Vero/4, Vero(ATCC CCL-81)/1	$10^{6.75}$ TCID <sub>50</sub> /200 $\mu$ l
Herpes Simplex Type 2 (HSV-2)	MS	VR-540	216463	Sheep choroid plexus/?, HeLa/?, PrRabK/7, Vero(ATCC CCL-81)/3	$10^{7.5}$ TCID <sub>50</sub> /200 $\mu$ l
Measles	Edmonston	VR-24	215236	HK/24, HuAm/40, MRC- 5/1, MRC-5(ATCC CCL- 171)/1	$10^8$ TCID <sub>50</sub> /ml

To test whether HSV-1 and HSV-2 and measles viruses obtained from the ATCC could replicate and be produced on PER.C6, passage number 46 cells were seeded in labtek chambers, coated with Poly-L-Lysine using methods known to persons skilled in the art, at  $10^5$  cells/well. Monkey derived Vero cells (obtained from ATCC) were cultured at passage number 137 and were used as positive controls and seeded at a density of  $2.5 \times 10^4$  cells/well. At day 0, when wells with PER.C6 cells were 60% and Vero cells 80% confluent, cells were infected with different moi's ( $10^{-3}$ ,  $10^{-2}$ ,  $10^{-1}$  and 1 TCID<sub>50</sub> per cell). At daily intervals upon infection, cells were fixed and assayed in immunofluorescence using FITC-conjugated type specific monoclonal antibodies using a kit (Imagen Herpes Simplex Virus (HSV) Type 1 and 2, (Dako) and FITC-conjugated antibodies against the HA and matrix protein of measles virus (measles IFA kit, Light diagnostics), following the procedures suggested by the manufacturer. The antisera are directed against HSV-1 and -2 and Measles virus antigens. The results summarized in **Fig.12** show that PER.C6 is permissive for HSV-1, HSV-2 and Measles virus infections. Furthermore, the kinetics suggest that these viruses replicate on PER.C6 in an moi-dependent manner.

Next it was investigated whether HSV-1, -2 and Measles virus could be propagated on PER.C6. To this end cells were infected with moi of 0.01, 0.1 and 1 TCID<sub>50</sub>/cell for HSV-1 (**Fig.13B**) and HSV-2 (**Fig.13A**) and an moi of 0.001 TCID<sub>50</sub>/cell for Measles virus (**Fig.13C**) (passage number 1). At the occurrence of almost complete cpe, cells and supernatants were harvested, quickly frozen in liquid N<sub>2</sub>, and thawed. After this, clarified supernatants were passaged blindly using approximately 100 µl, to PER.C6 (this is passage number 2). After reaching almost complete CPE again, a third passage (passage number 3) was performed in a similar manner. The

moi's of the passage number 2 and 3 were determined in retrospect by TCID<sub>50</sub> assays. The results of these experiments show that Herpes Simplex Virus type 1 and -2 and Measles viruses can be replicated on PER.C6 and that replication and propagation can even occur when moi's as low as 10<sup>-7</sup> are used.

#### **Example 8**

##### **Screening of rotavirus for replication on PER.C6.**

To test whether PER.C6 could also support the replication of a rotavirus, PER.C6 cells were infected with a Rhesus rotavirus (MMU 18006; ATCC#VR-954; strain S:USA:79:2; lot#2181153). PER.C6 cells (passage number 41) were cultured at a density of 1x10<sup>5</sup> per ml and Monkey derived Vero cells (obtained from ATCC, passage number 139) were cultured at a density of 2.5x10<sup>4</sup> per ml, and subsequently seeded in Labtek chambers, that had been pre-coated with poly-L-Lysine using methods known to persons skilled in the art. Cells were infected with an moi of 1 TCID<sub>50</sub>/cell of Rhesus rotavirus in the presence and absence of 2 µg/ml of trypsin-EDTA. After 90 min of infections, cells were washed with ExCell 525 medium and further incubated at 37°C at 10% CO<sub>2</sub> in a humidified atmosphere. On 5 consecutive days following infection, samples of supernatants were harvested, clarified from cells and cell debris by centrifugation at 2000 rpm in a table top centrifuge and analysed in an ELISA specific for rotavirus (IDEIA Rotavirus, Dako). The results depicted in Fig.14 clearly show that Rhesus rotavirus replicates on PER.C6.

#### **Example 9**

##### **Inhibition of Herpes virus propagation on PER.C6 by a purine nucleoside analogue.**

Acyclovir, 2-amino-1,9-dihydro-9-[(2-hydroethoxy)methyl]-6H-purin-6-one, is a synthetic purine analogue



with *in vivo* and *in vitro* inhibitory activity against herpes simplex virus types 1 and 2 (HSV-1, HSV-2) and against Varicella Zoster virus (VZV). The activity *in vitro* of acyclovir is the highest against HSV-1, followed by decreasing activities against HSV-2 and VZV. The mechanism of antiviral activity of this compound, which is highly selective, is based on its affinity for the enzyme thymidine kinase (TK) encoded by HSV and VZV. Acyclovir is converted by TK in acyclovir monophosphate, a nucleoside analogue that is further converted into diphosphate by cellular guanylate kinase and into triphosphate by a number of cellular enzymes. Acyclovir triphosphate stops the replication of herpes virus DNA in *in vitro* infected cell cultures by 1) inhibition of viral DNA polymerase, 2) chain termination of the viral DNA and 3) inactivation of the viral DNA polymerase. The differential sensitivity of HSV-1,2 and VZV is related to differences in phosphorylation efficiency by the viral TK's (De Clercq 2001).

To test the feasibility of PER.C6 for antiviral drug screening, the acyclovir/HSV system was used. A TCID<sub>50</sub> titration of both a HSV-1 as well as a HSV-2 stock virus produced on PER.C6 (titer 6000 TCID<sub>50</sub>/ml) was performed in either the absence or in the presence of different concentrations of acyclovir. PER.C6 cells were seeded in a 96-wells tissue culture plate at a concentration of  $1 \times 10^5$  cells/well, in normal culture medium, DMEM (Gibco) supplemented with heat inactivated FBS (origin US, Gibco) and 1 ml 4.9 M MgCl<sub>2</sub> (Sigma). The day after seeding, cells were pre-incubated with the appropriate acyclovir (20 mg/ml, Genthon) concentration being, 200, 20, 2, 0,2 and 0 µg/ml, diluted in normal culture medium, for 2 hours at 37°C and 10% CO<sub>2</sub>. A total of 20 wells were used per concentration. On a separate plate series of tenfold dilutions of HSV 1 (VR-539 ATCC, 3th passage on PER.C6) and HSV2 (VR-540 ATCC, 3th passage on PER.C6) were made

in the appropriate medium, supplemented with the respective concentrations of acyclovir according to the scheme in Table II. After the pre-incubation with acyclovir, the medium was removed from the cells, and 100  
 5  $\mu$ l of each of the viral dilutions with acyclovir present, were added to duplicate wells.

Table II. Titration scheme for testing acyclovir  
 10 virustatic activity against HSV-1 and HSV-2 replicating on PER.C6.

Viral input HSV-1: HSV-1 titer: 5000 TCID<sub>50</sub>/100  $\mu$ l  
 Viral input HSV-2: HSV-2 titer: 5000 TCID<sub>50</sub>/100  $\mu$ l  
 2x10<sup>5</sup> cells/well

Virus dilution	Input virus (TCID <sub>50</sub> )	Moi (TCID <sub>50</sub> /cell)
10 <sup>-1</sup>	10000	5x10 <sup>-2</sup>
10 <sup>-2</sup>	1000	5x10 <sup>-3</sup>
10 <sup>-3</sup>	100	5x10 <sup>-4</sup>
10 <sup>-4</sup>	10	5x10 <sup>-5</sup>
10 <sup>-5</sup>	1	5x10 <sup>-6</sup>
10 <sup>-6</sup>	0.1	5x10 <sup>-7</sup>
10 <sup>-7</sup>	0.01	5x10 <sup>-8</sup>
10 <sup>-8</sup>	0.001	5x10 <sup>-9</sup>

15

At day 6 post infection, CPE was scored in each of the wells and TCID<sub>50</sub> calculated according to the Karber method (1931), which is well known to persons skilled in the art. In addition, viable cells were scored with the MTS assay. Briefly, 25 µl of MTS substrate (Celltiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) was added to the wells. Metabolic active cells will reduce the MTS tetrazolium into a colored formazan product. The substrate was left on the cells for 2 hours at 37°C 10% CO<sub>2</sub>, followed by reading the OD<sub>490</sub> in an ELISA reader.

As shown in Fig.15, no toxicity of acyclovir for uninfected PER.C6 cells was noticed, which is in agreement with its mechanism of action requiring phosphorylation by herpes virus TK. A clear virustatic effect of acyclovir was observed for both HSV-1 and HSV-2 when propagated on PER.C6 cells. For HSV-1, as compared to the control, an almost 1000 fold TCID<sub>50</sub> reduction was observed at a concentration of 20 µg/ml of acyclovir (Fig.16 left panel). For HSV-2, a similar reduction of 1000 fold TCID<sub>50</sub> required a higher amount of acyclovir (Fig.16 right panel), which is in agreement with the differential sensitivity of HSV-2 for acyclovir. When scored for viable cells with MTS assay, which is well known to persons skilled in the art, comparable results were obtained (Fig.17A and B). Acyclovir was again found to be more effective at lower dosage in preventing cell death caused by HSV-1 than by HSV-2, now by using PER.C6 cells.

## REFERENCES

- Alrabiah FA and Sacks (1996). New anti herpes virus agents. Their targets and therapeutic potential. Drugs 52: 17-32.
- Bardsley-Elliott A and Plosker GL. (2000a) Nelfinavir: an update on its use in HIV infection. Drugs 59: 581-620.
- Bardsley-Elliott A and Perry CM. (2000b) Nevirapine: a review of its use in the prevention and treatment of paediatric HIV infection. Paediatr Drugs 2: 373-407.
- De Clercq E. (2001) Molecular targets for antiviral agents. J Pharmacol Exp Ther 297: 1-10.
- Dwyer DE and Kesson AM (1997) Advances in antiviral therapy. Curr Opin Pediatr 9: 24-30.
- Figgitt DP and Plosker GL. (2000) Saquinavir soft-gel capsule: an updated review of its use in the management of HIV infection. Drugs 60: 481-516.
- Herrero-Urbe L, Mann GF, Zuckerman AJ, Hockley D, Oxford JS. (1983) Replication of influenza A and B viruses in human diploid cells. J Gen Virol 64:471-475.
- Joly V, Moroni M, Concia E, Lazzarin A, Hirschel B, Jost J, Chiodo F, Bentwich Z, Love WC, Hawkins DA, Wilkins EG, Gatell AJ, Vetter N, Greenwald C, Freimuth WW, de Cian W. (2000) Delavirdine in combination with zidovudine in treatment of human immunodeficiency virus type 1-infected patients: evaluation of efficacy and emergence of viral resistance in a randomized, comparative phase III trial. The M/3331/0013B Study Group. Antimicrob Agents Chemother 44: 3155-3157.
- Karber G. Beitrag zur kollektiven behandlung pharmakologischer reihenversuche. (1931) Exp. Pathol. Pharmacol 162: 480-483.
- Keating MR (1999). Antiviral agents for non-human immunodeficiency virus infections. Mayo Clin Proc 74: 1266-1283.
- Li XQ, Gorelik E, Atchison RW, Ovejera A and Ho M. (1988) A new in vivo anti-viral assay using microencapsulated infected cell cultures. Antiviral Res 10: 179-192.
- Murray MA and Babe LM. (1999) Inhibitory effect of dibenzofuran and dibenzosuberol derivatives on rhinovirus replication in vitro; effective prevention of viral entry by dibenzosuberone. Antiviral Res 44: 123-131.

- Pastor-Anglada M, Felipe A and Casado FJ. (1998)  
Transport and mode of action of nucleoside derivatives  
used in chemical and antiviral therapies. Trends  
5 Pharmacol Sci 19: 424-30.
- Polyak SJ and Gerotto M (2000). The molecular basis for  
responsiveness to anti-viral therapy in hepatitis C.  
Forum (Genova) 10: 46-58.  
10
- Scott LJ and Perry CM. (2000) Delavirdine: a review of  
its use in HIV infection. Drugs 60: 1411-1444.
- Sidwell RW and Smee DF. (2000) In vitro and in vivo assay  
15 systems for study of influenza virus inhibitors.  
Antiviral Res 48: 1-16.
- Todd S, Anderson C, Jolly DJ and Craik CS. (2000) HIV  
protease as a target for retrovirus vector-mediated gene  
20 therapy. Biochim Biophys Acta 1477: 168-188.
- Ying C, De\_Clerq E and Neyts, J. (2000) Lamivudine,  
adefovir and tenofovir exhibit long-lasting anti-  
hepatitis B virus activity in cell culture. J Viral Hepat  
25 7: 79-83.
- International patent application No. PCT/NL00/00862,  
int. filing date 24.11.00, "Production of vaccines"

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## CLAIMS

(41)

1. A method for determining whether a compound influences a phase in the life cycle of a virus comprising providing a cell with said compound and with at least a fragment of said virus sufficient for performing said phase and determining whether said phase is influenced in said cell, said cell comprising a nucleic acid encoding an adenovirus early protein or a functional part, derivative and/or analogue of said adenovirus early protein.
2. A method according to claim 1, wherein said nucleic acid is integrated into the genome of said cell.
3. A method according to any one of claims 1 or 2, wherein said virus comprises an adenovirus, an enterovirus, a herpes virus, an orthomyxovirus, a paramyxovirus, a retrovirus, a rotavirus, a coronavirus, a flavivirus, a togavirus, a hepatitis causing virus, a pestivirus, a rhabdovirus or a Bunyaviridae virus.
4. A method according to any one of claims 1-3, wherein said cell is provided with an essentially intact virus.
5. A method according to any one of claims 1-4, wherein said cell is a human cell.
6. A method according to any one of claims 1-5, wherein said cell is derived from a kidney, a retina or amniotic fluid.
7. A method according to any one of claims 1-6, wherein said adenovirus early protein comprises an adenovirus early region 1 protein.
8. A method according to any one of claims 1-7, wherein said adenovirus early protein comprises an adenovirus early region 2 protein.
9. A method according to any one of claims 1-8, wherein determining whether a compound influences a phase

in the life cycle of a virus comprises examining the activity and/or amount of a cellular protein.

10. A method according to any one of claims 1-9, wherein determining whether a compound influences a phase  
5 in the life cycle of a virus comprises examining the interaction of said virus with said cell.

11. A method according to any one of claims 1-10, wherein determining whether a compound influences a phase in the life cycle of a virus comprises examining the  
10 activity and/or amount of said virus or a fragment thereof.

12. A method according to any one of claims 1-11, wherein determining whether a compound influences a phase in the life cycle of a virus comprises examining the  
15 viability of said cell.

13. Use of a cell, said cell comprising nucleic acid encoding an adenovirus early protein, for screening a library of compounds for the presence of a compound capable of influencing a phase in the life cycle of a  
20 virus capable of entering said cell.

14. Use of a cell according to claim 13, wherein said virus comprises an adenovirus, an enterovirus, a herpes virus, an orthomyxovirus, a paramyxovirus, a retrovirus, a rotavirus, a coronavirus, a flavivirus, a togavirus, a  
25 hepatitis causing virus, a pestivirus, a rhabdovirus or a Bunyaviridae virus.

15. A method for identifying a compound with antiviral activity comprising providing a cell with at least a fragment of a virus, said fragment capable of  
30 performing a step in the life cycle of said virus, providing said cell with a compound and determining whether said compound is capable of influencing said step in the life cycle of said virus, wherein said cell comprises a nucleic acid encoding an adenovirus early  
35 protein or a functional part, derivative and/or analogue of said adenovirus early protein and wherein said virus

comprises an adenovirus, an enterovirus, a herpes virus,  
 an orthomyxovirus, a paramyxovirus, a retrovirus, a  
 rotavirus, a coronavirus, a flavivirus, a togavirus, a  
 hepatitis causing virus, a pestivirus, a rhabdovirus or a  
 5 Bunyaviridae virus.

16. A method according to claim 15, wherein said  
 compound is part of a compound library.

17. A method according to any one of claims 15 or 16,  
 wherein said method is performed in a high-throughput  
 10 setting.

18. A method according to any one of claims 15-17,  
 wherein said method further comprises isolating said  
 compound.

19. A method for identifying a compound with  
 15 antiviral activity comprising

- providing a cell from a first collection of  
 cell cultures with at least a fragment of a first virus,  
 said fragment capable of performing a step in the life  
 cycle of said first virus,

- 20 - providing said cell from a first collection of  
 cell cultures with a compound and

- determining whether said compound is capable of  
 influencing said step in the life cycle of said first  
 virus,

25 said method further comprising

- providing a cell from a second collection of  
 cell cultures with at least a fragment of a second virus,  
 said fragment capable of performing a step in the life  
 cycle of said second virus,

- 30 - providing said cell from a second collection of  
 cell cultures with a second compound

- and determining whether said second compound is  
 capable of inhibiting said step in the life cycle of said  
 second virus,

35 wherein said cells from said first and said second  
 collection comprise a nucleic acid encoding an adenovirus



early protein and wherein said first and said second library of compounds may be the same or different.

20. A method for determining the effect of a compound on a phase in the life cycle of a virus comprising the steps of

- culturing a cell, said cell otherwise capable of supporting said phase in the life cycle of a virus, in the presence of said virus under conditions otherwise conducive to said phase in the life cycle of said virus and in the absence of said compound,

- examining the effect of the absence of said compound on said phase in the life cycle of said virus.

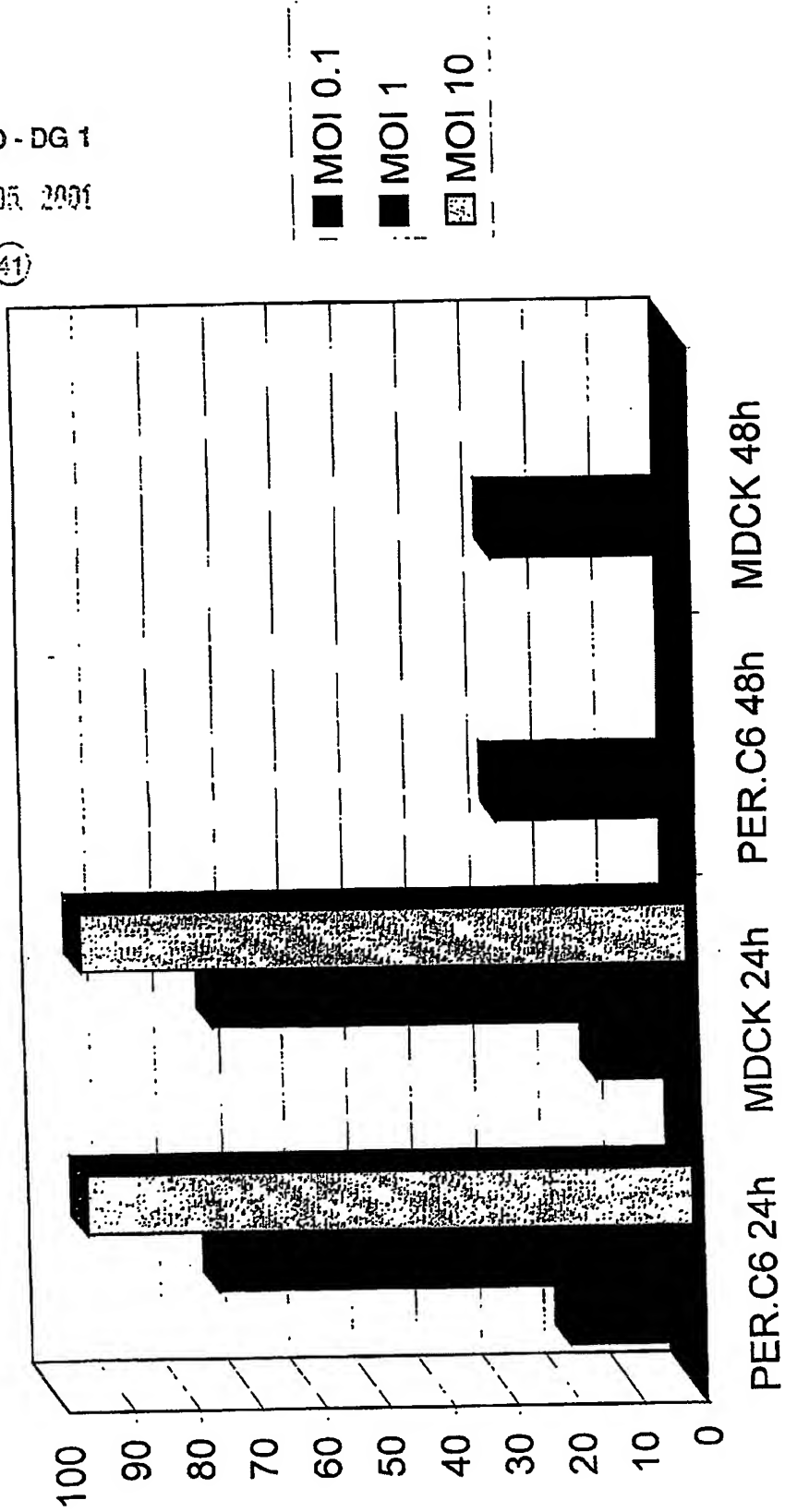
21. A method according to claim 20, wherein said compound is a natural constituent of said cell.

22. A method according to claim 21, wherein said natural constituent is a receptor protein, or a fragment thereof, for said virus.

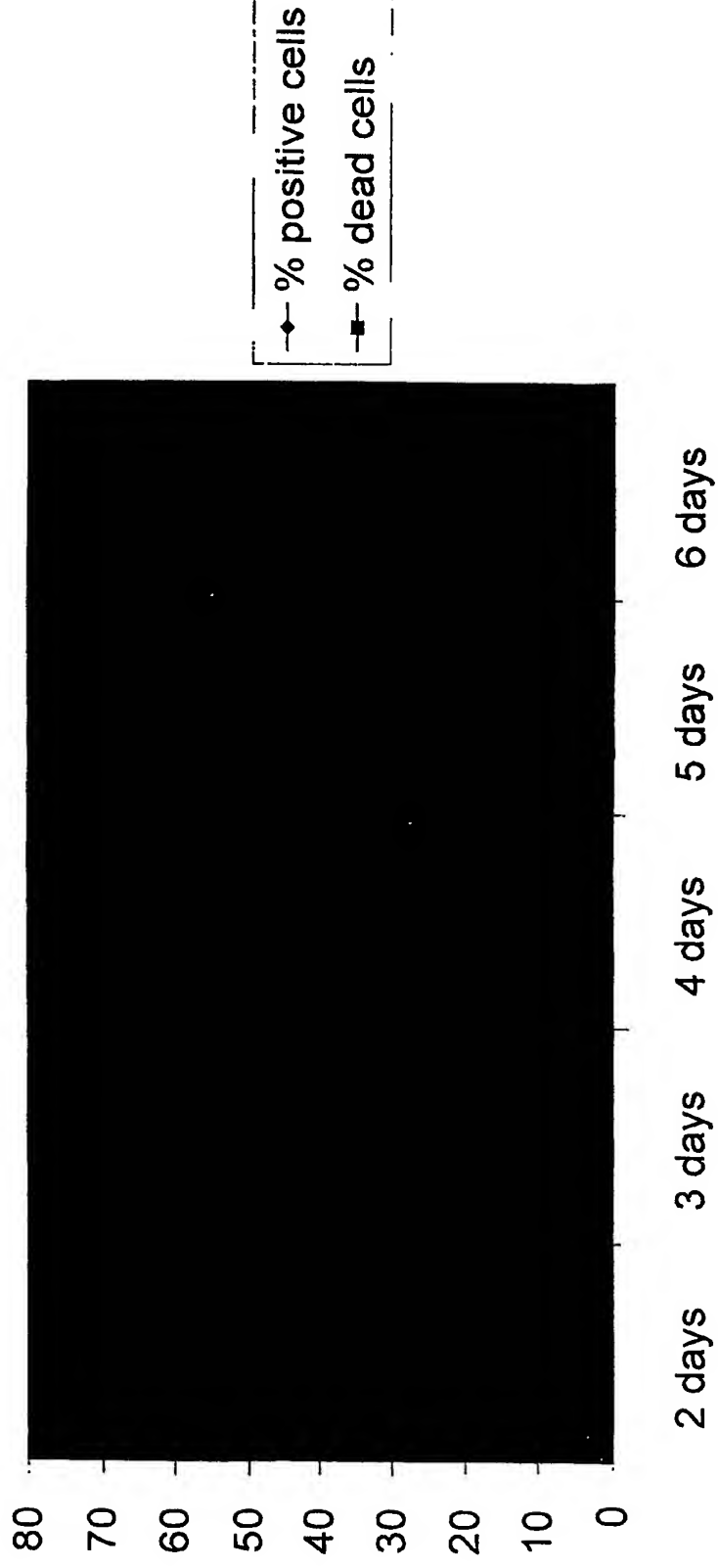
23. A method according to any one of claims 21-22, wherein said cell is comprised in a set of clones of cells or a library of cells, said cells comprising a gene being effectively blocked from being expressed.

24. A method according to any one of claims 20-23, wherein said cell comprises a nucleic acid encoding an adenovirus early protein or a functional part, derivative and/or analogue of said adenovirus early protein.

Figure 1



**Figure 2A**  
**MOI 10-3**



**Figure 2B**  
**MOI 10-4**

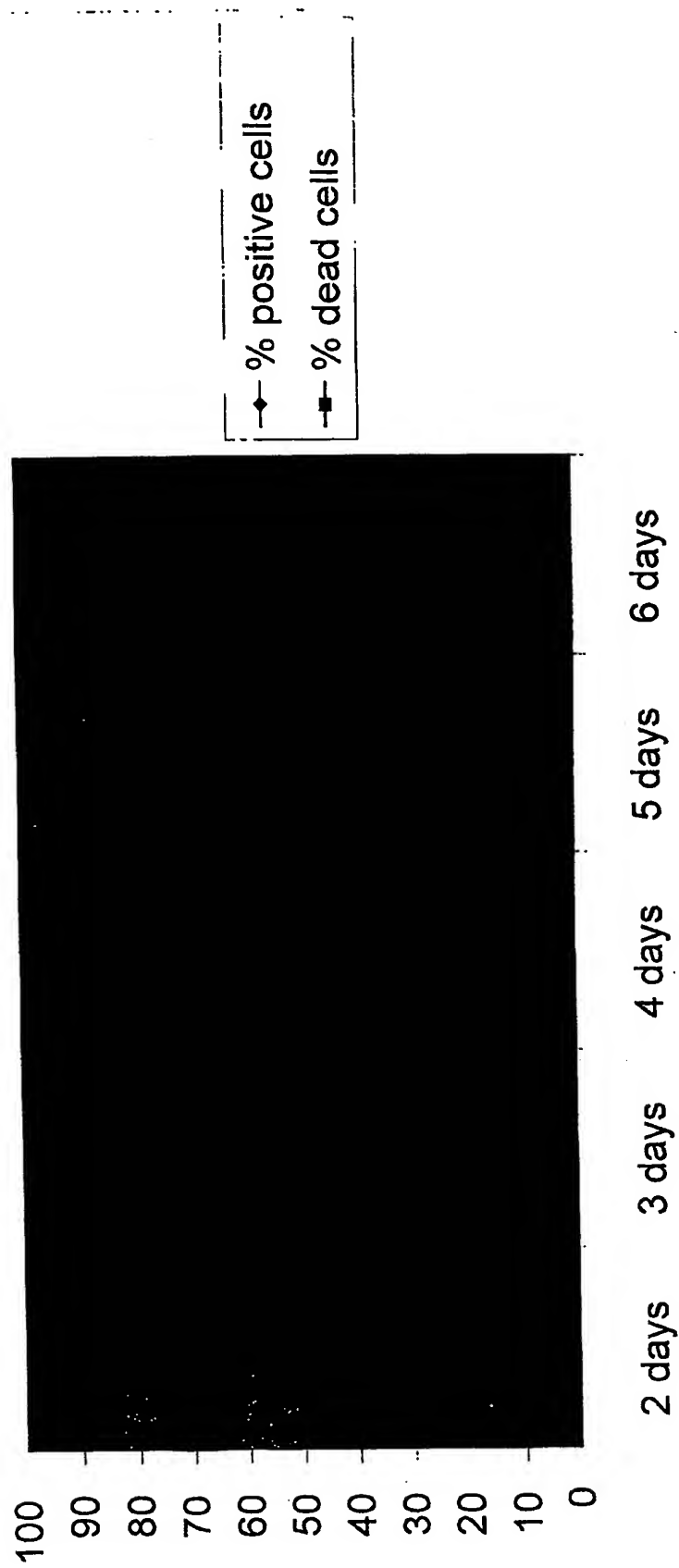
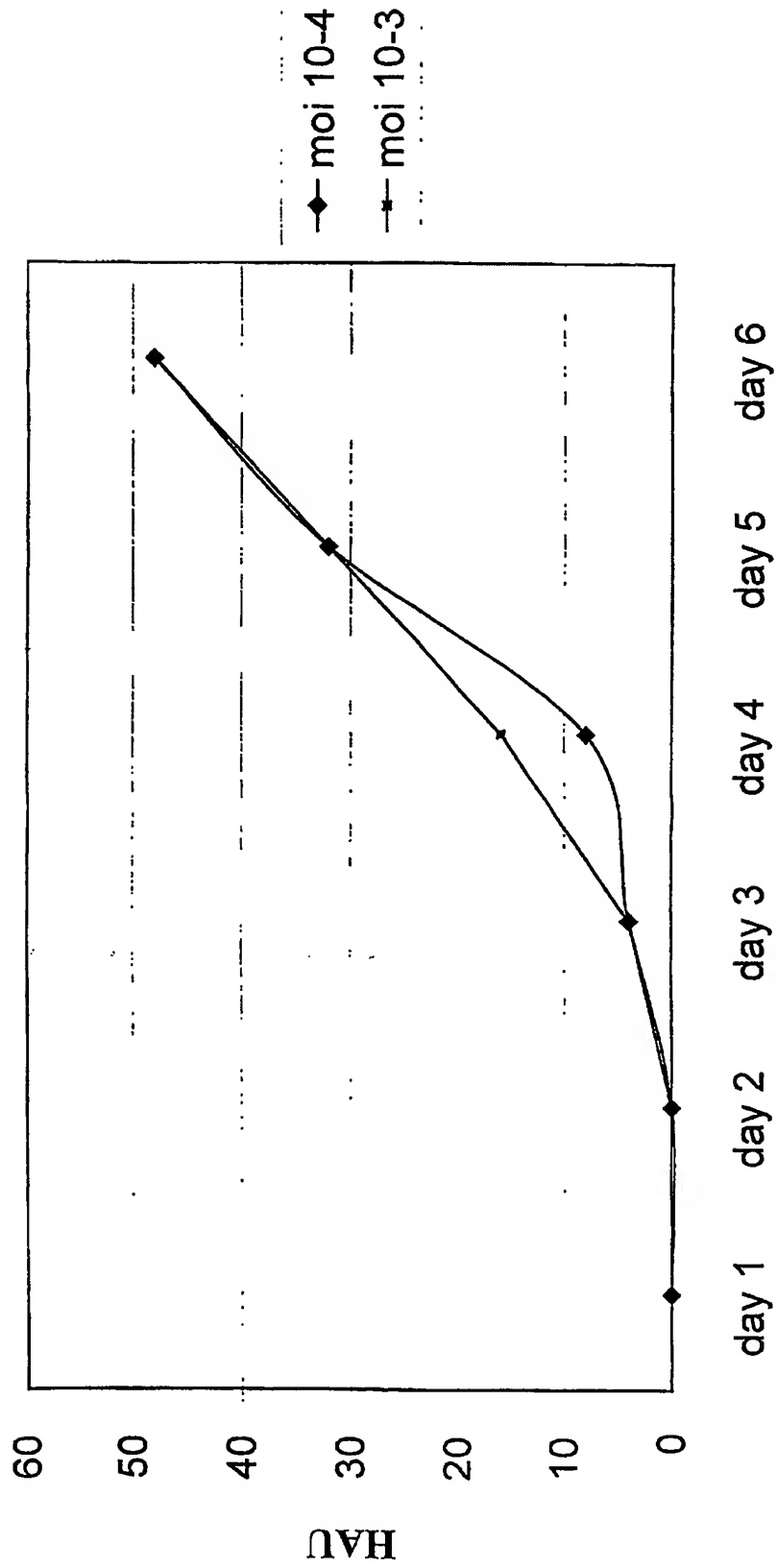


Figure 3



**Figure 4A**  
**A/Beijing/262/95**

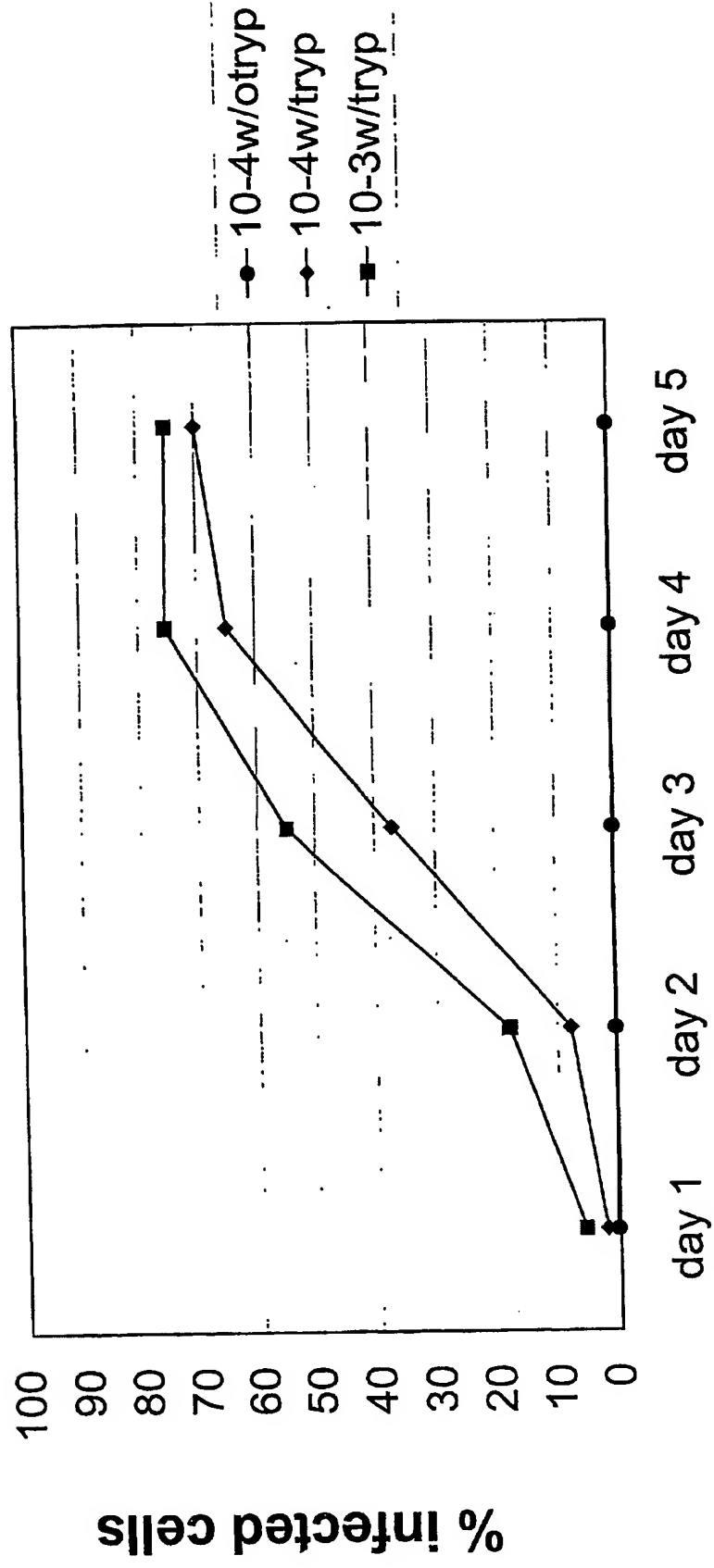
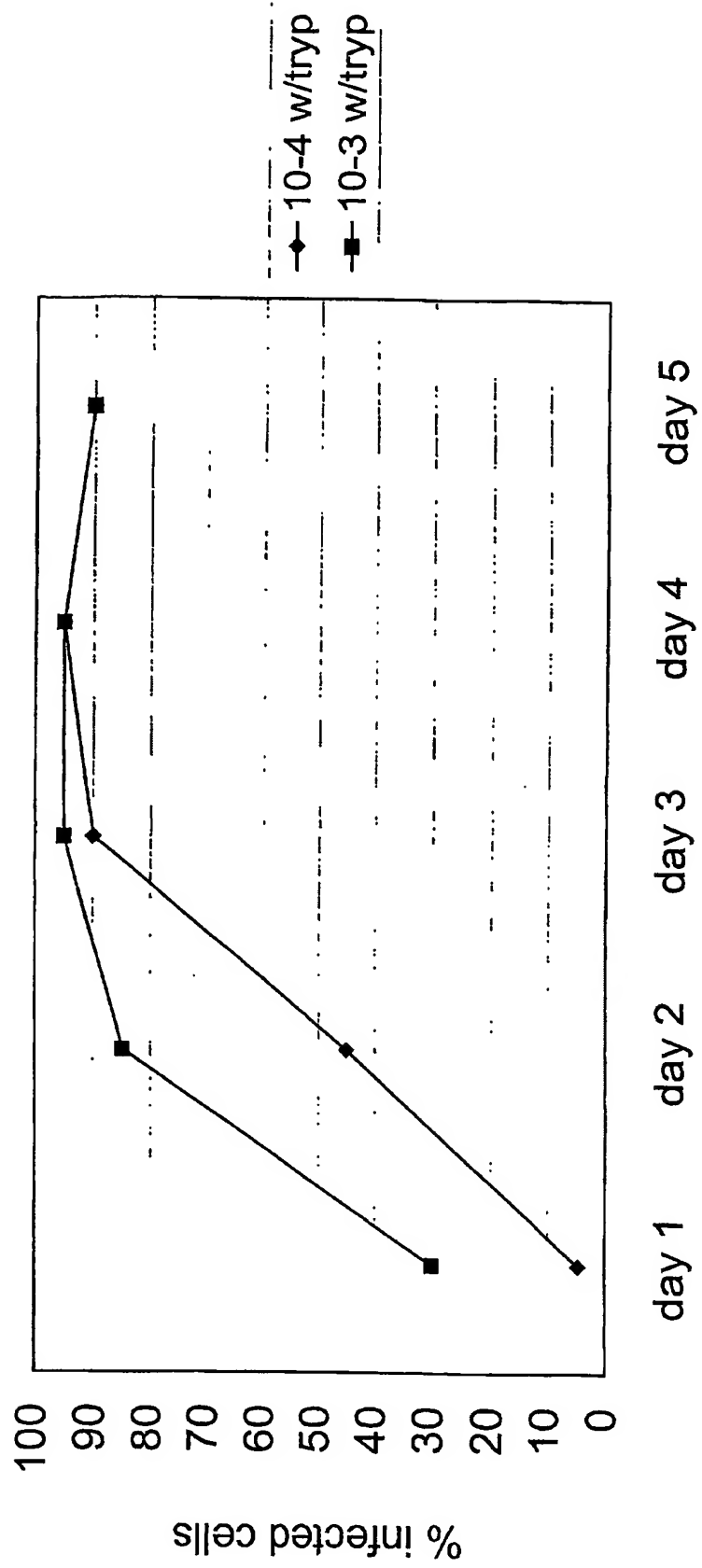


Figure 4B  
X-127



**Figure 5A**  
**A/Beijing/262/95**

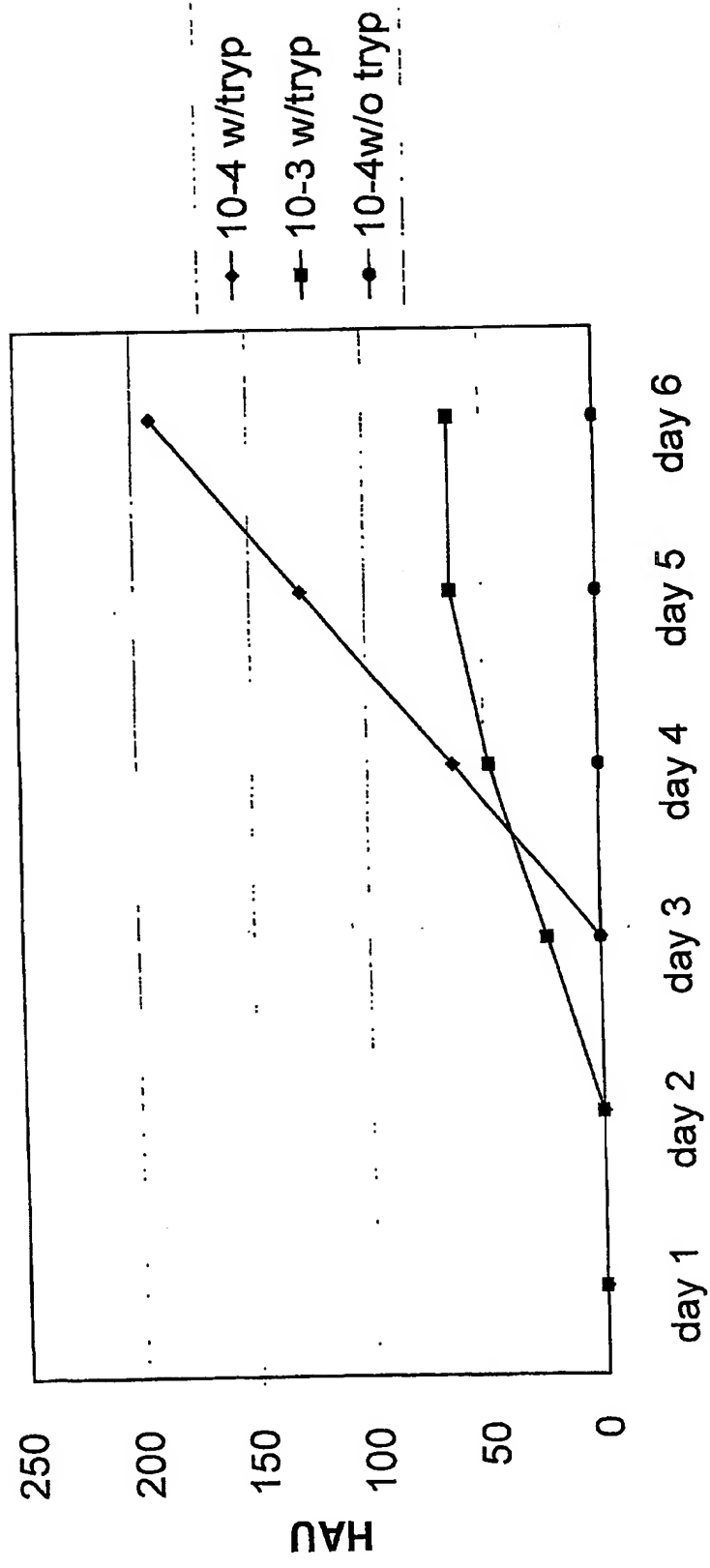




Figure 5B  
X-127

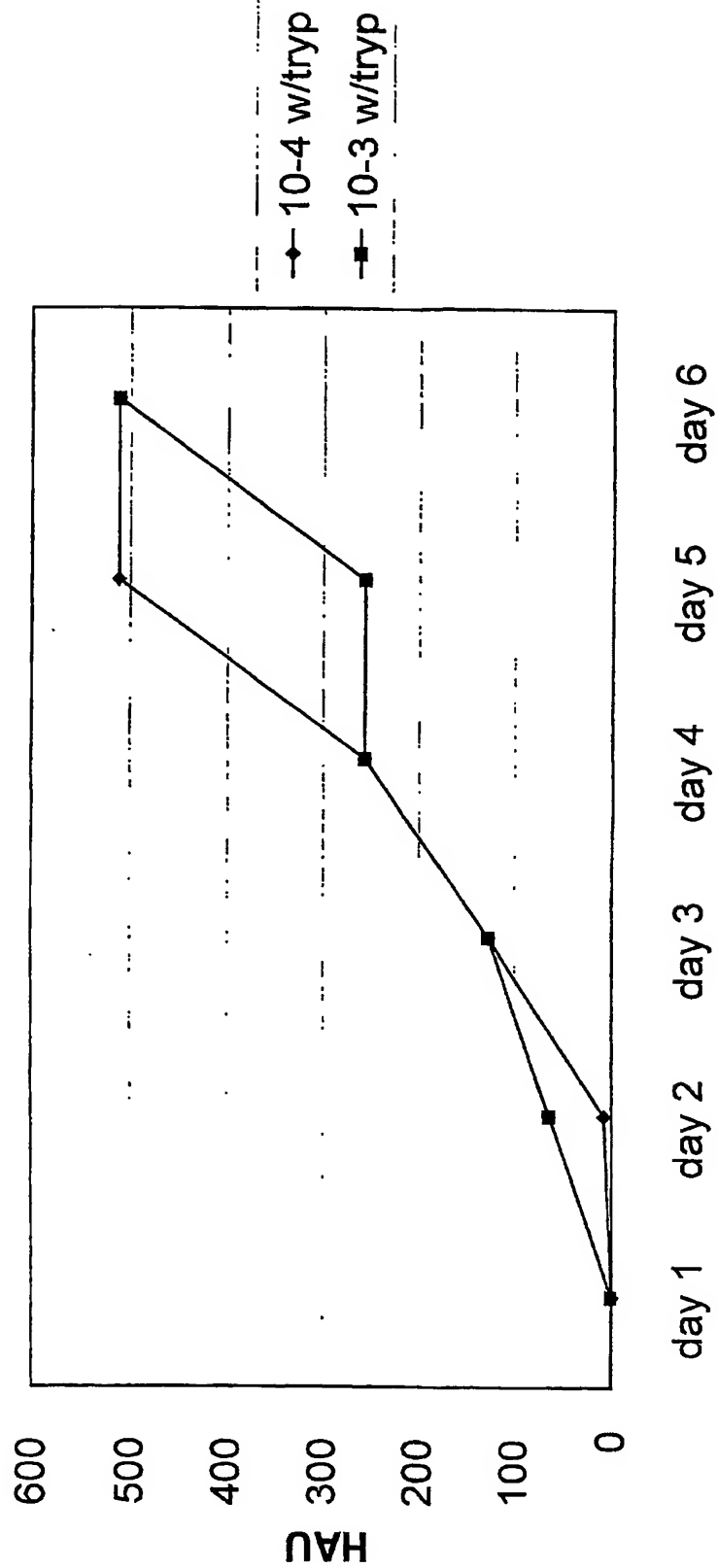


Figure 6

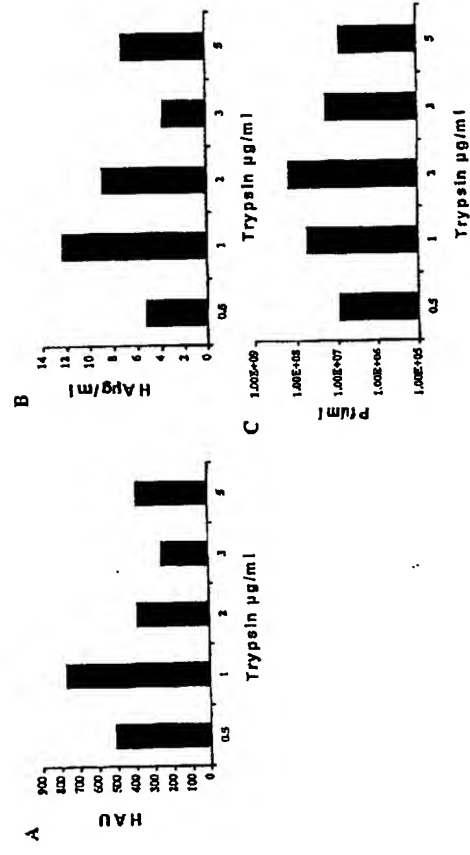


Figure 7

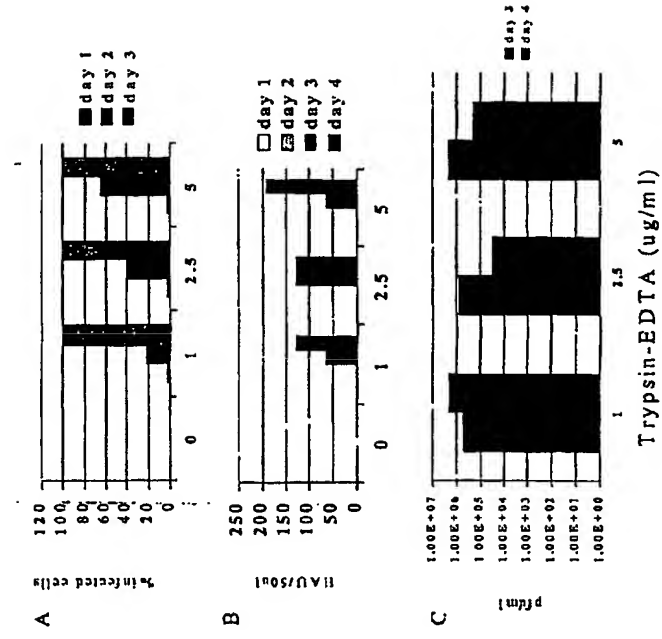


Figure 8

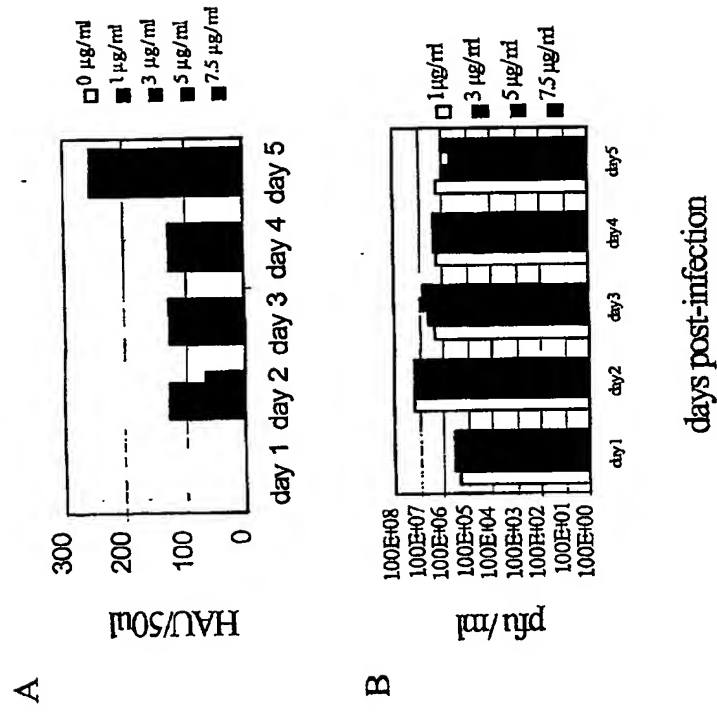


Figure 9.

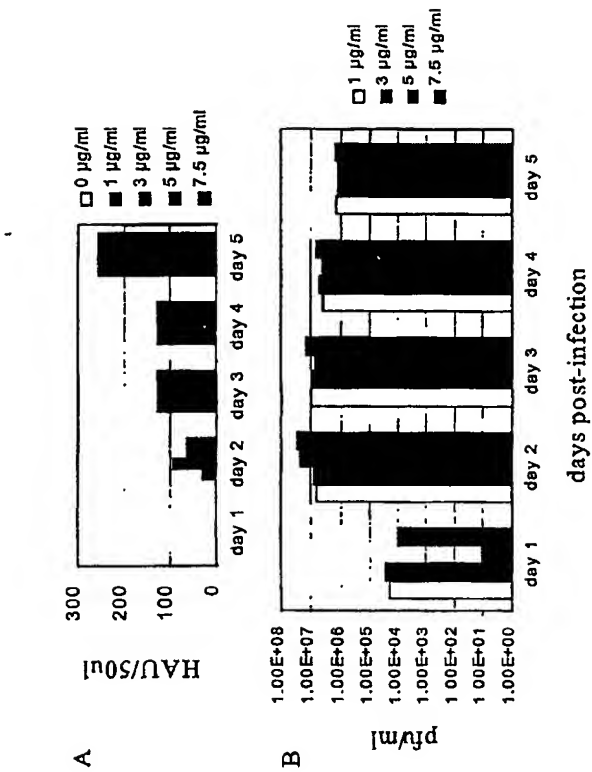


Figure 10

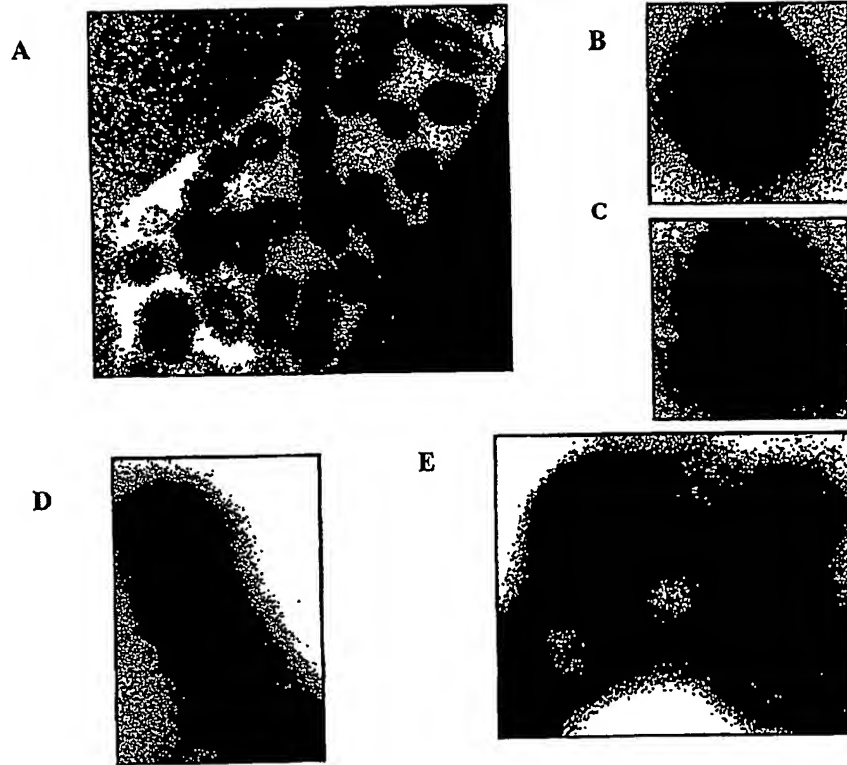


Figure 11

Influenza virus on PER.C6™

- H3N2 strains  
A/Sydney/5/97\*  
A/Johannesburg/33/94\*  
A/Nanchang/833/85\*  
A/Wuhan/359/95\*  
A/Moscow/10/99\*  
A/Panama/2007/99
  - B strains  
B/Harbin/7/94  
B/Yamanashi/166/98  
B/Beijing/184/93\*  
B/Shandong/7/97

- H1N1 strains  
A/Beijing/262/85  
X-127 A/Beijing reassortant  
A/New Caledonia/20/89  
A/Johannesburg/282/96  
A/Texas/36/91
  - Avian strains  
A/Duck/Singapore-Q/F119-3/97\*
- \* Difficult growth on eggs

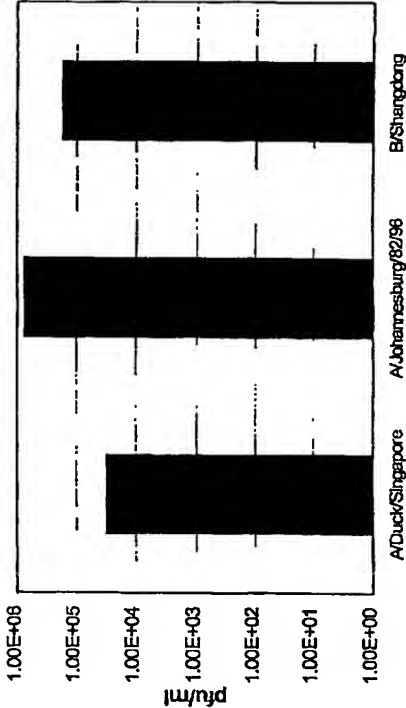


Figure 12A

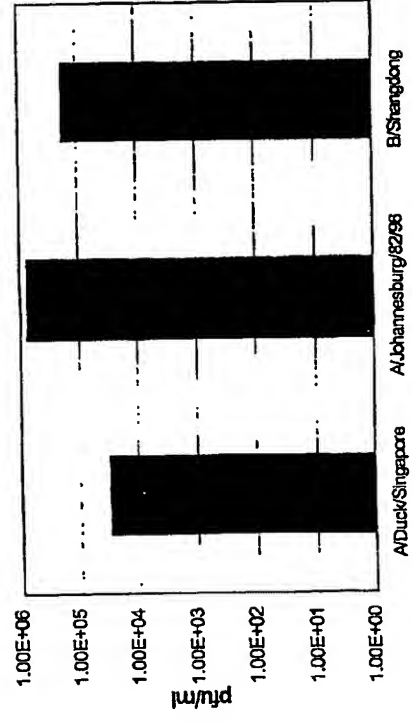




Figure 12 B - E

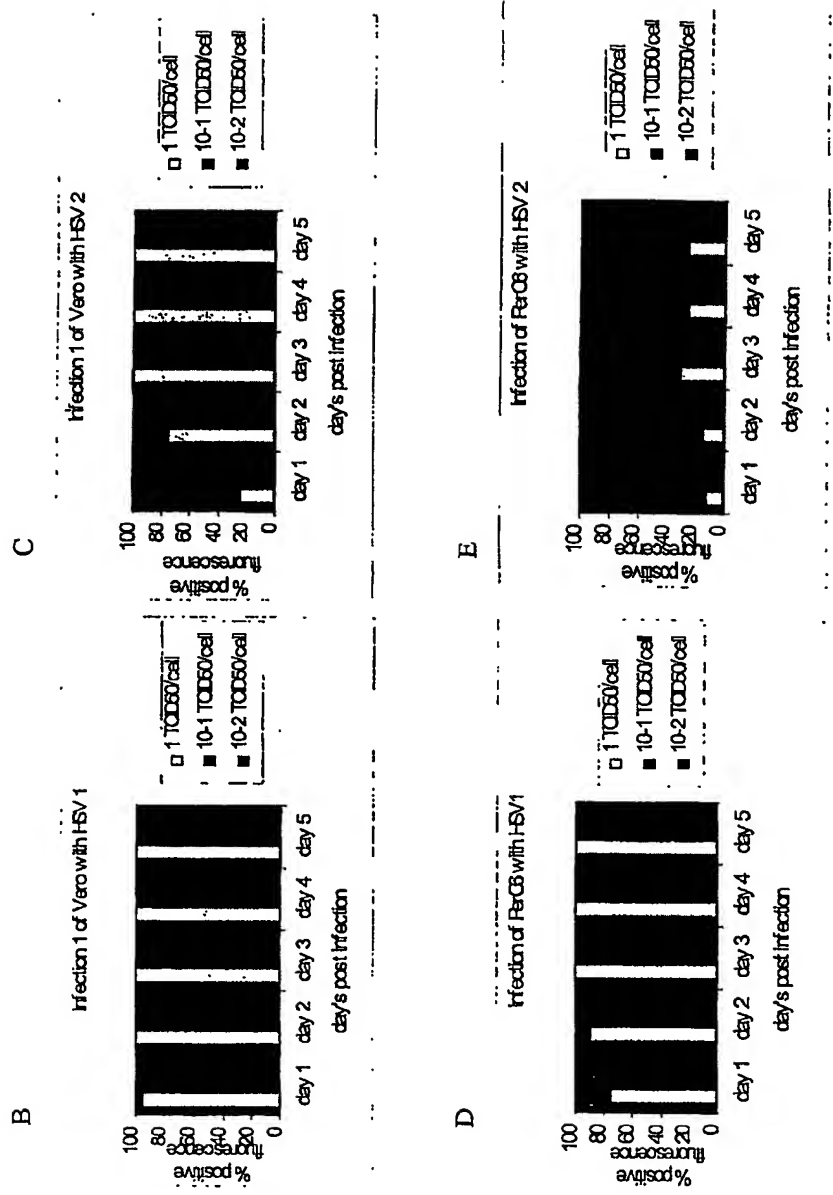


Figure 13A

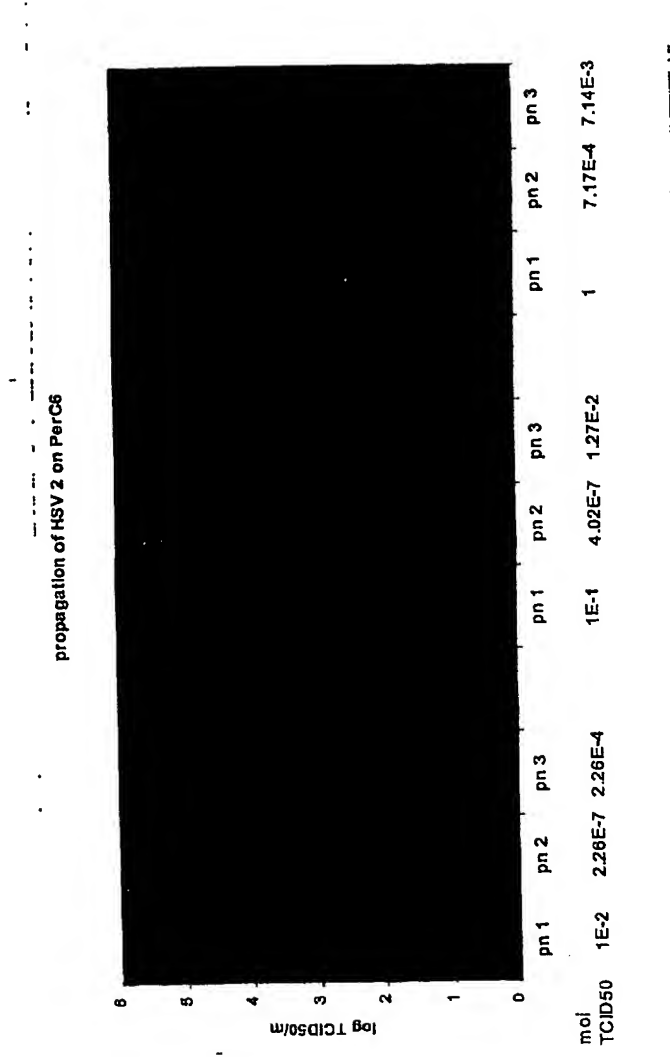


Figure 13B

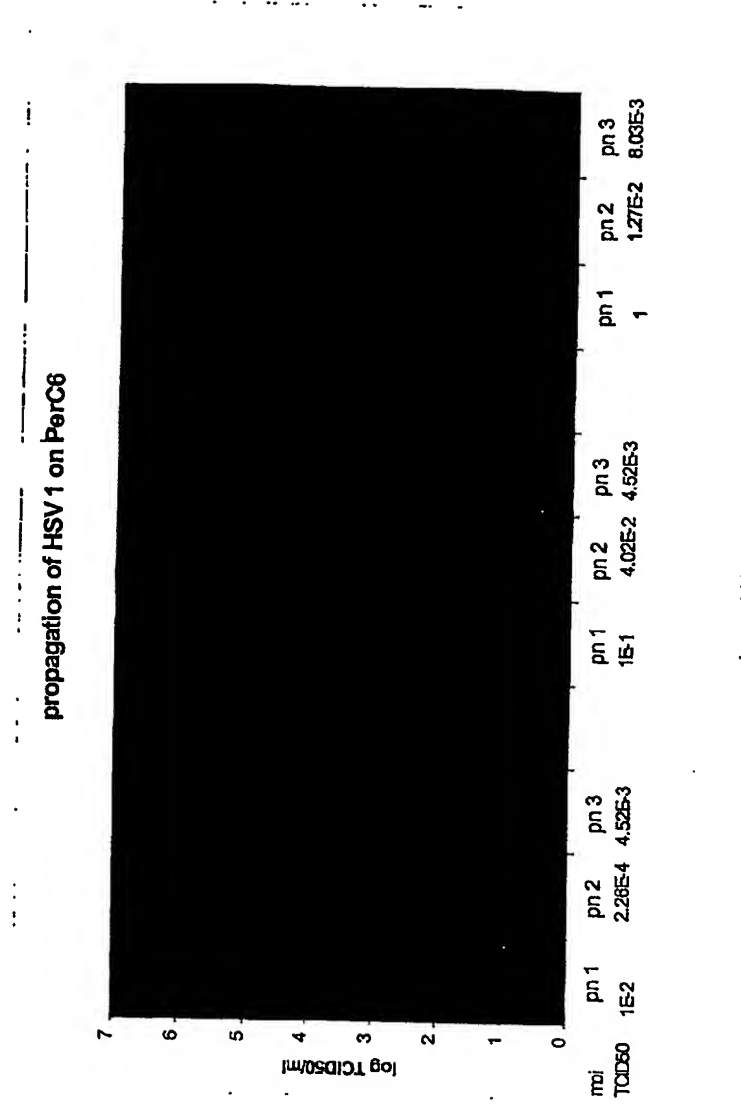


Figure 13C

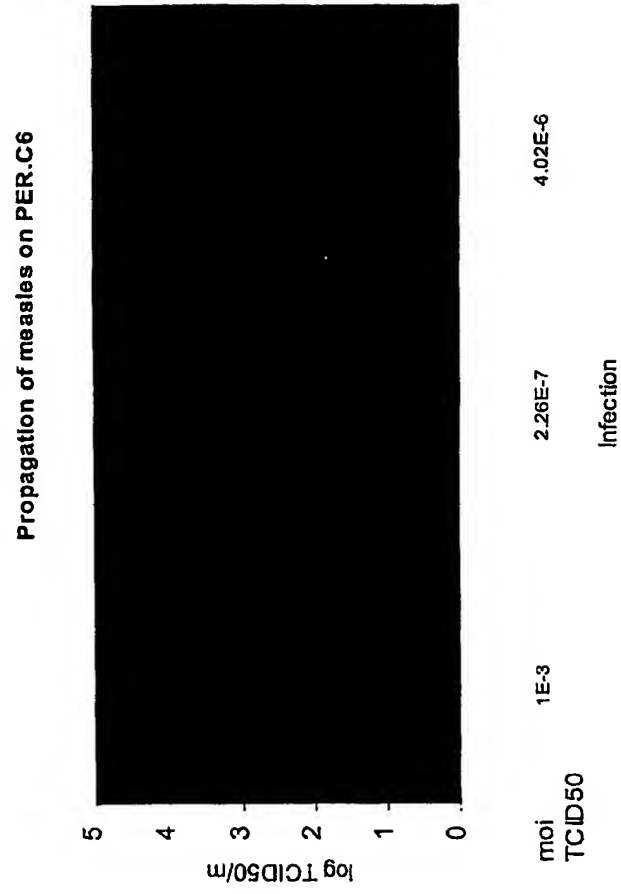


Figure 14A

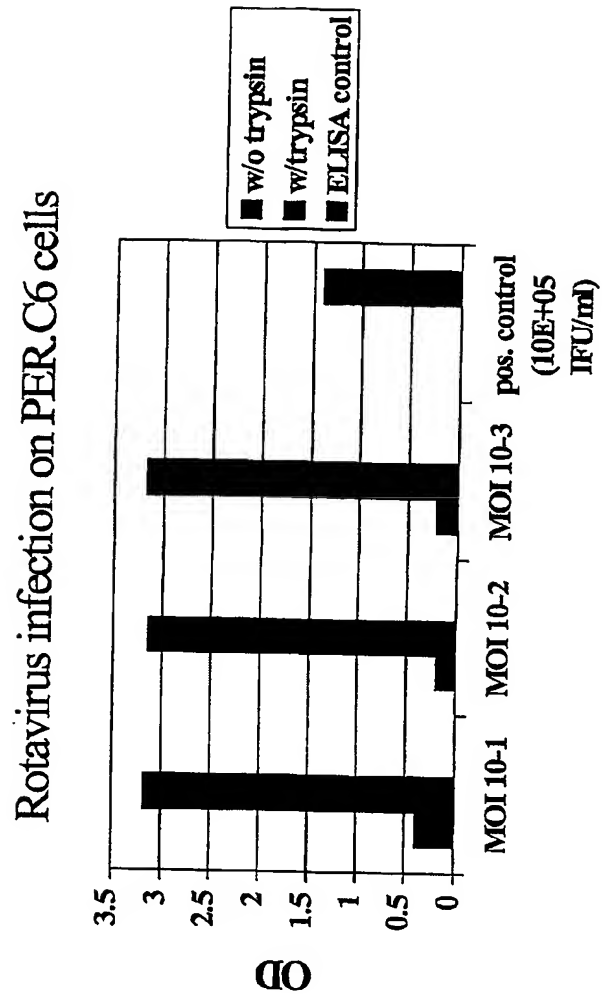


Figure 14B

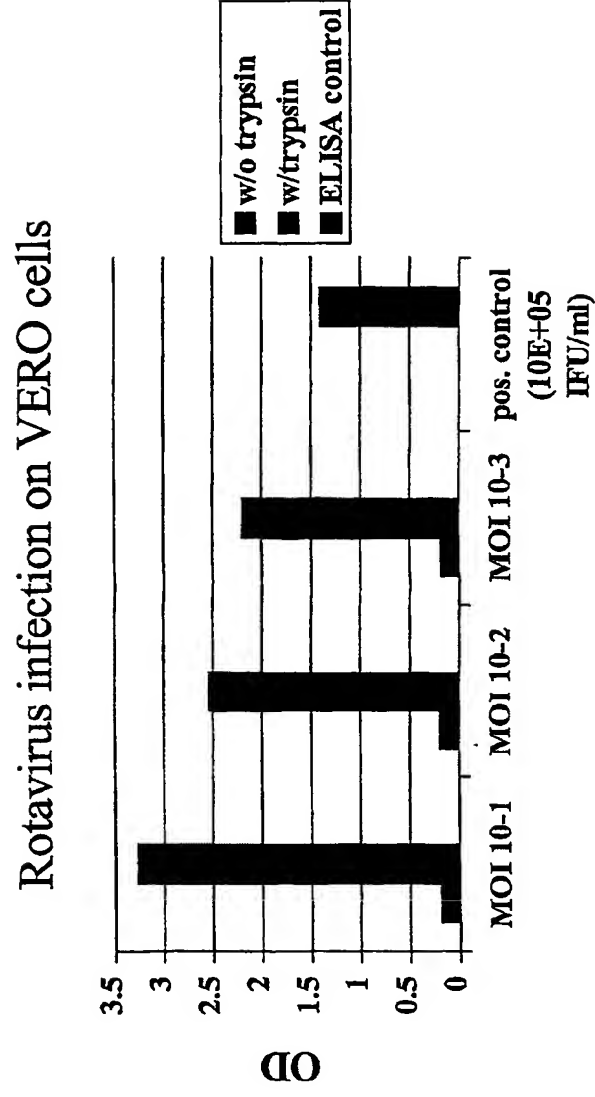


Figure 15

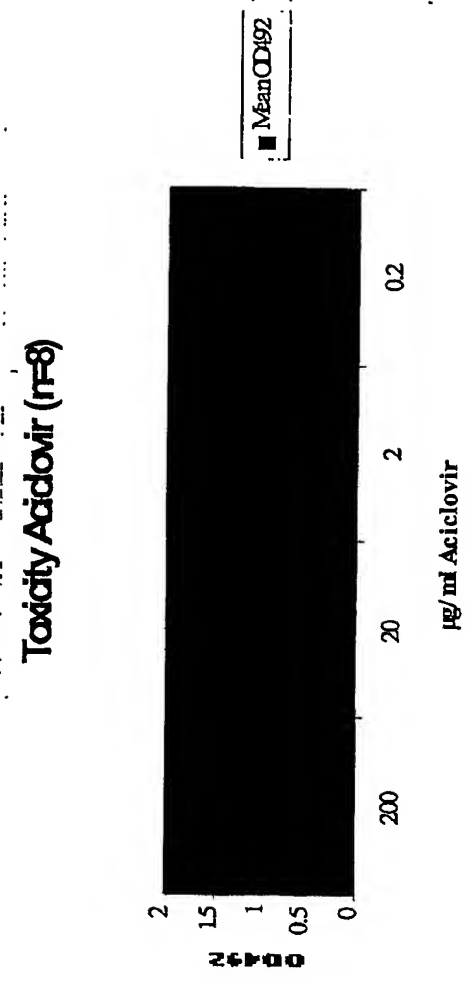


Figure 16

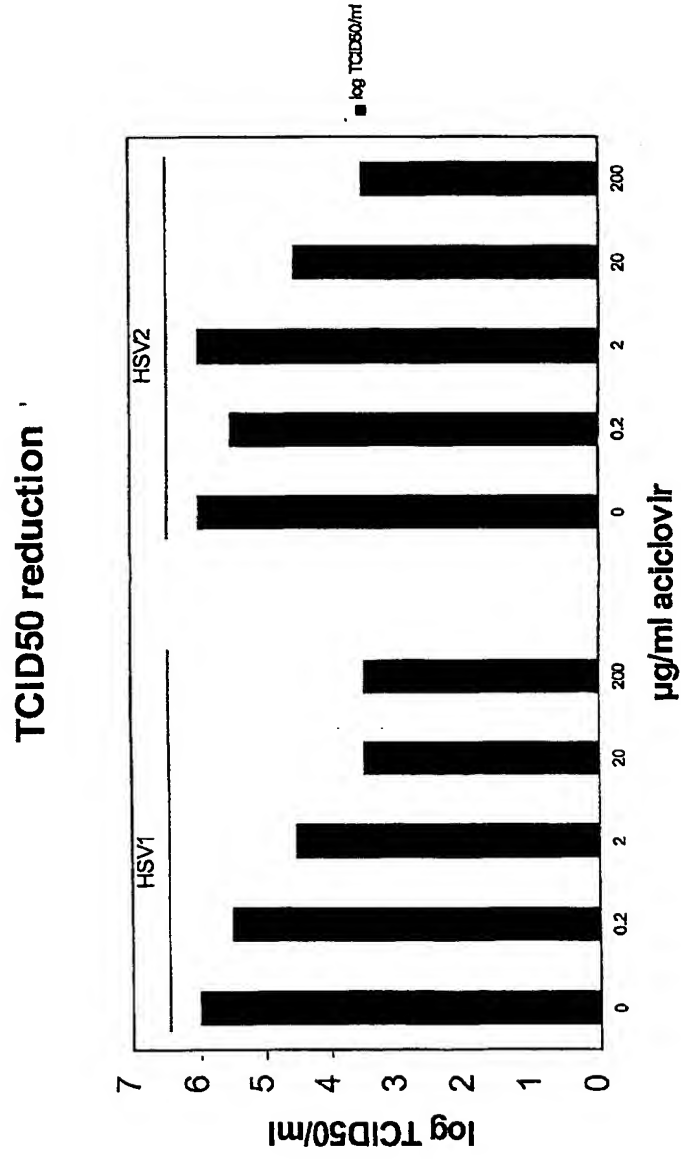




Figure 17A

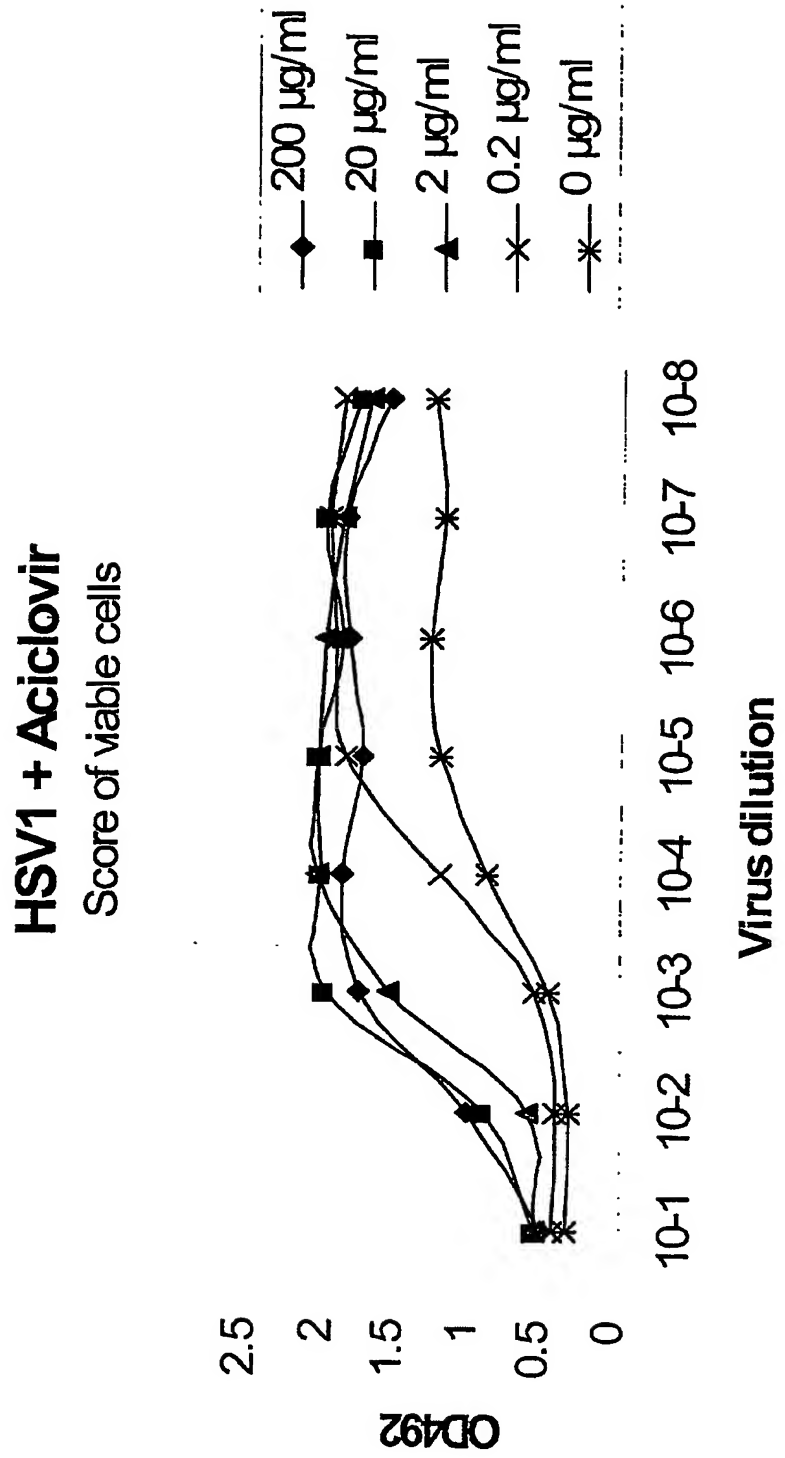
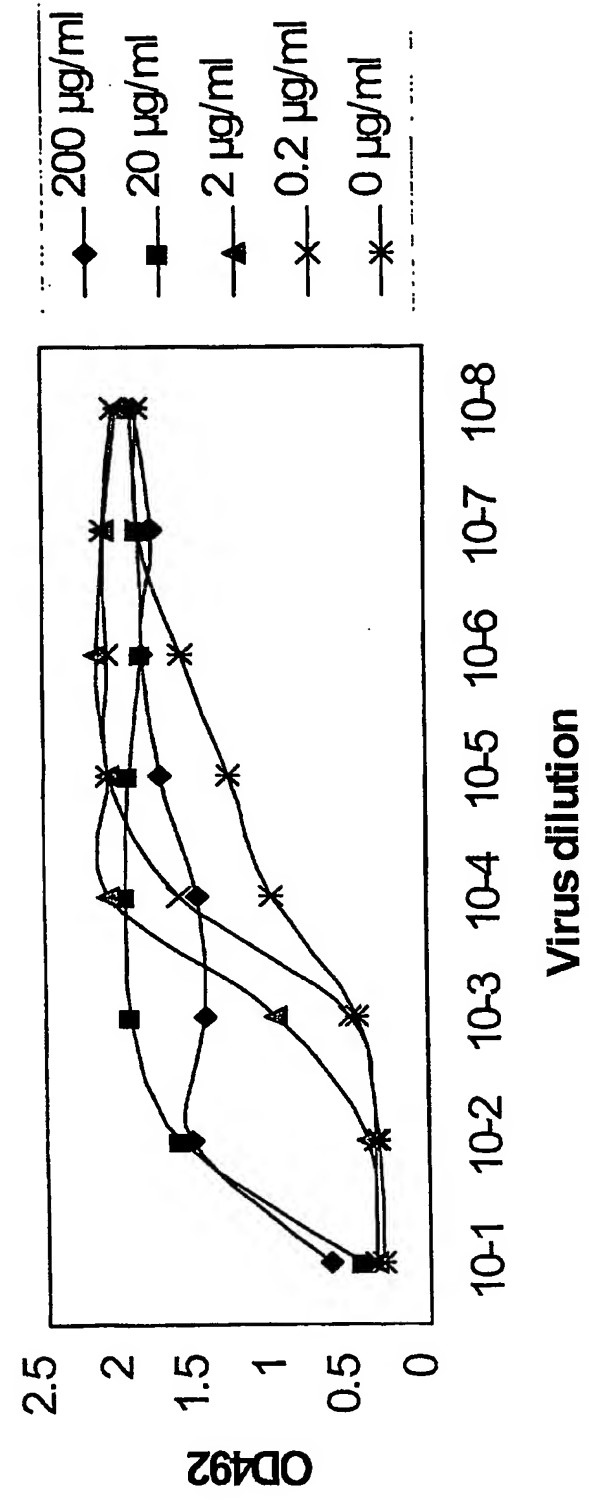


Figure 17B

# **HSV2 + Aciclovir,** Score of viable cells



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